Gene Therapy Protocols
Helicobacter Pylori Protocols, edited by Christopher L. Clayton and Harry T. Mobley, 1997
Human Cell Culture Protocols, edited by Gareth E. Jones, 1996
Antisense Therapeutics, edited by Sudhir Agrawal, 1996
Prion Diseases, edited by Harry F. Baker and Rosalind M. Ridley, 1996
Molecular Diagnosis of Cancer, edited by Finbarr Cotter, 1996
Molecular Diagnosis of Genetic Diseases, edited by Rob Elles, 1996
Preface

In the last few years, significant advances have been made in the area of gene therapy for both genetic and acquired diseases. Improvement in gene transfer methods has allowed for development of gene therapy protocols for the treatment of diverse types of diseases, including metabolic, cardiovascular, and autoimmune diseases, as well as cancer. For example, clinical trials for gene therapy of cancer, cystic fibrosis, ADA deficiency, and arthritis, among others, have been initiated in recent years. It is likely that, in the near future, gene therapy will become a common form of treatment for many different types of diseases. Gene therapy takes advantage of recent advances in many areas of molecular and cell biology, including the identification of new therapeutic genes, improvement in both viral and nonviral gene delivery systems, better understanding of gene regulation, and improvement in cell isolation and transplantation. Because of the different and complex techniques involved in achieving successful gene-mediated therapies, it is difficult for scientists to perform all methods required for gene delivery and subsequent in vivo gene expression.

This *Gene Therapy Protocols* volume in the *Methods in Molecular Medicine* series provides scientists with detailed methods for working with gene transfer systems, and methods for gene transfer to specific tissue types either in vivo or ex vivo. The first part of the book is concerned with the methods for working with specific delivery systems, including adenoviruses, retroviruses, herpes viruses, polioviruses, and papilloma viruses, as well as nonviral systems, such as liposomes and DNA conjugates. The latter part of the book is concerned with the application of the gene delivery methods to specific tissues, such as airway epithelium, liver, hematopoietic cells, keratinocytes, tumors, and synovium. In addition, methods for the application of specific therapeutic systems, such as ribozymes and tumor suppressor genes for the treatment of cancer, are presented.

Because of its wide scope, *Gene Therapy Protocols* should be of general interest to undergraduate and postgraduate students and to both basic and clinical researchers.

*Paul D. Robbins*
Contents

Preface ............................................................................................................. v
Contributors ..................................................................................................... xi

1 Methods for Gene Transfer Using DNA-Adenovirus Conjugates,
   David T. Curiel ................................................................. 1

2 Methods for the Construction and Propagation of Recombinant
   Adeno-Associated Virus Vectors,
   Jeffrey S. Bartlett and Richard J. Samulski .......................... 25

3 Generation of High-Titer, Helper-Free Retroviruses by Transient
   Transfection,
   Warren S. Pear, Martin L. Scott, and Garry P. Nolan ...... 41

4 Methods for the Construction of Retroviral Vectors and the
   Generation of High-Titer Producers,
   Isabelle Rivière and Michel Sadelain .............................. 59

5 Development of Replication-Defective Herpes Simplex Virus Vectors,
   David Krisky, Peggy Marconi, William F. Goins,
   and Joseph C. Glorioso ................................................. 79

6 Methods for the Use of Poliovirus Vectors for Gene Delivery,
   Casey D. Morrow, David C. Ansardi, and Donna C. Porter .... 103

7 Methods for the Construction of Human Papillomavirus Vectors,
   Saleem A. Khan and Francis M. Sverdrup ...................... 117

8 Methods for Liposome-Mediated Gene Transfer to the Arterial Wall,
   Elizabeth G. Nabel, Zhi-yong Yang, Hong San, Dianne P. Carr,
   and Gary J. Nabel .......................................................... 127

9 Methods for Targeted Gene Transfer to Liver Using DNA-Protein
   Complexes,
   Mark A. Findeis, Catherine H. Wu, and George Y. Wu ...... 135

10 Methods for the Use of Retroviral Vectors for Transfer of the CFTR
    Gene to Airway Epithelium,
    John C. Olsen, Larry G. Johnson, and James R. Yankaskas ...... 153

11 Methods for Adenovirus-Mediated Gene Transfer to Airway
    Epithelium,
    John F. Engelhardt ............................................................. 169
Contents

12 Methods for Retrovirus-Mediated Gene Transfer to Fetal Lung,
   Bruce R. Pitt, Margaret A. Schwarz, and Richard D. Bland .......... 185
13 In Situ Retrovirus-Mediated Gene Transfer into the Liver,
   Nicolas Ferry, Sophie Branchereau, Jean-Michel Heard, and
   Olivier Danos ........................................................... 195
14 Methods for Delivery of Genes to Hepatocytes In Vivo Using
   Recombinant Adenovirus Vectors,
   Darlene Barr and Mark A. Kay ........................................... 205
15 Methods for Producing High Titer, Pantropic Retroviral Vectors
   for Gene Transfer into Leukemic T-Cells,
   Jane C. Burns, Jiing-Kuan Yee, and Alice L. Yu ..................... 213
16 Methods for Efficient Retrovirus-Mediated Gene Transfer to Mouse
   Hematopoietic Stem Cells,
   John W. Belmont and Roland Jurecic .................................. 223
17 Methods for Retrovirus-Mediated Gene Transfer into Primary
   T-Lymphocytes,
   Michel Sadelain .................................................................. 241
18 Methods for Retrovirus-Mediated Gene Transfer to CD34+ Enriched
   Cells,
   Alfred B. Bahnson, Maya Nimgaonkar, Edward D. Ball, and
   John A. Barranger .......................................................... 249
19 Methods for the Use of Genetically Modified Keratinocytes
   in Gene Therapy,
   Sabine A. Eming and Jeffrey R. Morgan ............................... 265
20 Methods for Particle-Mediated Gene Transfer into Skin,
   Ning-Sun Yang, Dennis E. McCabe, and William F. Swain ........ 281
21 Methods for Liposome-Mediated Gene Transfer to Tumor Cells
   In Vivo,
   Gary J. Nabel, Zhi-yong Yang, and Elizabeth G. Nabel ............ 297
22 Methods for Retrovirus-Mediated Gene Transfer to Tumor Cells,
   Elizabeth M. Jaffee ........................................................... 307
23 Cationic Liposome-Mediated Gene Transfer to Tumor Cells In Vitro
   and In Vivo,
   Kyonghee Son, Frank Sorgi, Xiang Gao, and Lea Huang .......... 329
24 Methods for the Use of Cytokine Gene-Modified Tumor Cells
   in Immunotherapy of Cancer,
   Zhihai Qin and Thomas Blankenstein .................................. 339
# Contents

25 Methods for Generation of Genetically Modified Fibroblasts for Immunotherapy of Cancer,  
*Elaine M. Elder, Michael T. Lotze, and Theresa L. Whiteside* ...... 349  
26 Methods for Gene Transfer to Synovium,  
*Richard Kang, Paul D. Robbins, and Christopher H. Evans* ....... 357  
27 Methods for Adenovirus-Mediated Gene Transfer to Synovium In Vivo,  
*Blake J. Roessler* .................................................................................. 369  
28 Methods for the Use of Stromal Cells for Therapeutic Gene Therapy,  
*Joel S. Greenberger* ............................................................................. 375  
29 Suppression of the Human Carcinoma Phenotype by an Antioncogene Ribozyme,  
*Toshiya Shitara and Kevin J. Scanlon* ................................................... 391  
30 Methods for Cancer Gene Therapy Using Tumor Suppressor Genes,  
*Wei-Wei Zhang and Jack A. Roth* ......................................................... 403  
Index ............................................................................................................ 419
Contributors

DAVID C. ANSARDI • Department of Microbiology, University of Alabama, Birmingham, AL
ALFRED B. BAHNSON • Department of Human Genetics, University of Pittsburgh, PA
EDWARD D. BALL • Department of Medicine, University of Pittsburgh, PA
DARLENE BARR • Division of Transplantation, Department of Surgery, University of Washington, Seattle, WA
JOHN A. BARRANGER • Department of Human Genetics, University of Pittsburgh, PA
JEFFREY S. BARTLETT • Gene Therapy Center, University of North Carolina, Chapel Hill, NC
JOHN W. BELMONT • Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX
RICHARD D. BLAND • Division of Lung Biology, Childrens Research Center, University of Utah School of Medicine, Salt Lake City, UT
THOMAS BLANKENSTEIN • Max-Delbrück-Centrum for Molecular Medicine, Berlin, Germany
SOPHIE BRANCHEREAU • Laboratoire Rétrovirus et Transfert Génétique, Institut Pasteur, Paris, France
JANE C. BURNS • Department of Pediatrics, School of Medicine, University of California, San Diego, CA
DIANNE P. CARR • Departments of Internal Medicine and Biological Chemistry, Howard Hughes Medical Institution, University of Michigan Medical Center, Ann Arbor, MI
DAVID T. CURIEL • University of Alabama, Birmingham, AL
OLIVIER DANOS • Laboratoire Rétrovirus et Transfert Génétique, Institut Pasteur, Paris, France
ELAINE M. ELDER • Immunologic Monitoring and Diagnostic Laboratory, University of Pittsburgh Cancer Institute, Pittsburgh, PA
SABINE A. EMING • Research Center, Shriners Burns Institute, Cambridge, MA
JOHN F. ENGELHARDT • Institute of Human Gene Therapy, Philadelphia, PA
CHRIS H. EVANS • Department of Molecular Genetics and Biochemistry, University of Pittsburgh Medical Center, Pittsburgh, PA
NICOLAS FERRY • Centre Regional de Lutte Contre le Cancer Eugene Marquis, Rennes, France
MARK A. FINDEIS • Pharmaceutical Peptides, Inc., Cambridge, MA
XIANG GAO • Laboratory of Drug Targeting, Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA; Current address: Division of Pulmonary Medicine, Vanderbilt University, Nashville, TN
JOSEPH C. GLORIOSO • Department of Molecular Genetics and Biochemistry, University of Pittsburgh Medical School, Pittsburgh, PA
WILLIAM F. GOINS • Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA
JOEL S. GREENBERGER • University of Pittsburgh Medical Center, Presbyterian Hospital, Pittsburgh, PA
JEAN-MICHEL HEARD • Laboratoire Rétrovirus et Transfert Génétique, Institut Pasteur, Paris, France
LEAF HUANG • Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA
ELIZABETH M. JAFFEE • Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD
LARRY G. JOHNSON • Cystic Fibrosis/Pulmonary Research and Treatment Center, Department of Medicine, University of North Carolina, Chapel Hill, NC
ROLAND JURECIC • Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX
RICHARD KANG • Orthopedic Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA
MARK A. KAY • Division of Medical Genetics, Department of Medicine, University of Washington, Seattle, WA
SALEEM A. KHAN • Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA
DAVID KRISKY • Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA
MICHAEL T. LOTZE • Cancer Institute, Department of Surgery, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA
PEGGY MARCONI • Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA
DENNIS E. MCCABE • Agracetus, Middletown, WI
Contributors

JEFFREY R. MORGAN • Research Center, Shriners Burns Institute, Cambridge, MA
CASEY D. MORROW • Department of Microbiology, University of Alabama, Birmingham, AL
ELIZABETH G. NABEL • Internal Medicine and Physiology, Cardiovascular Research Center, University of Michigan Medical Center, Ann Arbor, MI
GARY J. NABEL • Departments of Internal Medicine and Biological Chemistry, Howard Hughes Medical Institution, University of Michigan Medical Center, Ann Arbor, MI
MAYA NIMGAONKAR • Department of Medicine, University of Pittsburgh, PA
GARRY P. NOLAN • Department of Pharmacology, Stanford University, Stanford, CA
JOHN C. OLSEN • Cystic Fibrosis/Pulmonary Research and Treatment Center, Department of Medicine, University of North Carolina, Chapel Hill, NC
WARREN S. PEAR • Department of Biology, Massachusetts Institute of Technology, Cambridge, MA; Current address: Department of Pathology, University of Pennsylvania Medical Center, Philadelphia, PA
BRUCE R. PITT • Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA
DONNA C. PORTER • Department of Microbiology, University of Alabama, Birmingham, AL
ZHIGHAI QIN • Max-Delbrück-Centrum for Molecular Medicine, Berlin, Germany
ISABELLE RIVIÈRE • New York University Medical Center, New York
PAUL D. ROBBINS • Department of Molecular Genetics and Biochemistry, University of Pittsburgh, PA
BLAKE J. ROESSLER • Department of Internal Medicine, Division of Rheumatology, University of Michigan Medical Center, Ann Arbor, MI
JACK A. ROTH • Department of Tumor Biology, University of Texas M. D. Anderson Science Center, Houston, TX
MICHEL SADELAIN • Department of Human Genetics, Memorial Sloan-Kettering Cancer Center, New York
RICHARD J. SAMULSKI • Gene Therapy Center, University of North Carolina, Chapel Hill, NC
HONG SAN • Departments of Internal Medicine and Biological Chemistry, Howard Hughes Medical Institution, University of Michigan Medical Center, Ann Arbor, MI
KEVIN J. SCANLON • City of Hope National Medical Center, Duarte, CA
MARGARET A. SCHWARZ • Babies Hospital, Columbia University College of Physicians and Surgeons, New York, NY
MARTIN L. SCOTT • Department of Biology, Massachusetts Institute of Technology, Cambridge, MA
TOSHIYA SHITARA • Department of Urology, Kitasato University Hospital, Kitasato, Japan
KYONGHEE SON • Laboratory of Drug Targeting, Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA; Current address: Department of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ
FRANK SORGI • Laboratory of Drug Targeting, Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA
FRANCIS M. SVERDRUP • Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA
WILLIAM F. SWAIN • Agracetus, Middletown, WI
THERESA L. WHITESIDE • Cancer Institute, Department of Pathology, and Department of Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA
CATHERINE H. WU • Division of Gastroenterology, University of Connecticut Health Center, Farmington, CT
GEORGE Y. WU • Division of Gastroenterology, University of Connecticut Health Center, Farmington, CT
NING-SUN YANG • Cancer Gene Therapy, Agracetus, Middletown, WI; Department of Pathology and Laboratory Medicine, University of Wisconsin Medical School, Madison, WI
ZHI-YONG YANG • Departments of Internal Medicine and Biological Chemistry, Howard Hughes Medical Institution, University of Michigan Medical Center, Ann Arbor, MI
JAMES R. YANKASKAS • Department of Medicine, Cystic Fibrosis/Pulmonary Research and Treatment Center, University of North Carolina, Chapel Hill, NC
JIING-KUAN YEE • Department of Pediatrics, City of Hope, Duarte, CA
ALICE L. YU • Department of Pediatrics, University of California at San Diego School of Medicine, La Jolla, CA
WEI-WEI ZHANG • Section of Thoracic Molecular Oncology, Department of Thoracic and Cardiovascular Surgery, University of Texas M. D. Anderson Science Center, Houston, TX
Methods for Gene Transfer Using DNA-Adenovirus Conjugates

David T. Curie

1. Introduction

Strategies have been developed to accomplish gene delivery via the receptor-mediated pathway employing molecular conjugate vectors (1–13). As cells possess endogenous pathways for internalization of macromolecules, the utilization of these pathways for the purpose of DNA delivery represents a strategy that potentially allows certain practical advantages. In this regard, these cellular internalization pathways can be highly efficient. For example, internalization of the iron transport protein transferrin can be on the order of thousands of molecules per minute per cell (14,15). These pathways thus represent a potentially efficient physiologic method to transport DNA across the cell membrane of eukaryotic cells. To accomplish gene transfer via receptor-mediated endocytosis, a vehicle must be derived that allows DNA entry into these cellular pathways. For this purpose, molecular conjugate vectors have been derived. These vector agents consist of two linked functional domains: a DNA-binding domain to transport the DNA as part of the vector complex, and a ligand domain to target a cellular receptor that allows entry of the conjugate-DNA complex into a receptor-mediated endocytosis pathway. For incorporating DNA into the complex for gene delivery, binding must be achieved in a nondamaging, reversible manner. For this linkage, an electrostatic association between the binding domain and the nucleic acid is accomplished. To achieve this, the DNA binding domain is comprised of a polycationic amine, such as poly(L)lysine. This can associate with the negatively charged DNA in an electrostatic, noncovalent manner. To achieve entry of the complex through a receptor-mediated pathway, a ligand for the target cell is utilized. The ligand domain is covalently linked to the polylsine to create the molecular conjugate vector.
The ligand domain may be a native or synthetic cell surface receptor ligand, an antireceptor antibody, or other agent that allows specific association with target cell membranes.

The interaction of the DNA with its binding domain serves not only to attach it to the molecular conjugate vector, but also to condense it into a compact circular toroid (5). In this configuration, the ligand domain is presented on the exterior of the complex. By virtue of its surface location, the ligand is free to recognize its target receptor on the cell surface membrane. Thus, after binding by means of the ligand domain, the conjugate-DNA complex is internalized by the receptor-mediated pathway. In this schema, the initial localization after internalization is within the cellular endosome. The conjugate-DNA complex may then achieve DNA delivery to the target cell nucleus, where expression of the transported heterologous sequences may occur. Alternatively, the complex may traffic to lysosomal pathways, which would result in degradation of the conjugate-DNA complex. Thus, by exploiting an endogenous cellular entry pathway, the conjugate-DNA complex achieves target cell internalization. After internalization, the complex can be subject to multiple possible fates; however, optimally, DNA delivery to the nucleus permits heterologous gene expression.

The delivery of genes by the receptor-mediated pathway offers certain unique features and potentials (4,16). Because the system is synthetic, the capacity exists to prepare large amounts of the conjugate vector. As delivery is by a physiologic cellular pathway, toxicity associated with membrane perturbation is circumvented. Thus, the potential exists to administer the vector on a repetitive, or continuous basis. Importantly, the marked plasticity of the system allows the potential to derive a vector with the properties of cell-specific targeting. That is, through choice of the ligand domain, it is possible to selectively target cells possessing receptors for that ligand. This would potentially allow employment for those gene therapy applications requiring cell-specific delivery of therapeutic genes. Finally, the molecular conjugate vector system is devoid of viral gene elements. It would thus be devoid of the potential safety hazards deriving from the presence of viral gene sequences and functions.

Gene delivery by the receptor-mediated endocytosis pathway was first described by Wu et al. (2). To selectively target hepatocytes, efforts were directed toward accomplishing cellular internalization through a receptor unique to this cell type. In this regard, hepatocytes possess unique receptors for recognition and clearance of asialoglycoproteins. This receptor is a constituent of a high efficiency internalization pathway specific to hepatocytes. To target through this receptor, asialoorosomucoid, a physiologic ligand for this receptor, was chemically conjugated to poly(L)lysine. The resulting conjugates could form complexes with DNA that could be shown to accomplish gene delivery
Fig. 1. Gene transfer via the receptor-mediated endocytosis pathway. A bifunctional molecular conjugate is employed to bind DNA and transport it via cellular macromolecular transport mechanisms. The molecular conjugate vector consists of a DNA-binding domain, comprised by a cationic polylysine moiety that is covalently linked to a ligand for a cell surface receptor, in this case transferrin. Plasmid DNA bound to the polylysine moiety of the conjugate undergoes marked condensation to yield an 80- to 100-nm toroid with surface-localized transferrin molecules. When the transferrin ligand domain is bound by its corresponding cell surface receptor, the conjugate is internalized by the receptor-mediated endocytosis pathway, cotransporting bound DNA. Escape from the cell vesicle system is achieved by a fraction of the internalized conjugate-DNA complex to achieve nuclear localization where heterologous gene expression occurs.

specifically through the asialoglycoprotein receptor of hepatocytes. Utilizing this schema, it was shown that selective delivery to hepatocytes could be accomplished both in vitro as well as after in vivo delivery of the conjugates (1,2,17). Utilizing a similar strategy, Birnstiel et al. developed a molecular conjugate system for achieving DNA delivery through the transferrin internalization pathway (Fig. 1; refs. 3,7). In this context, transferrin is internalized by a receptor-mediated endocytosis mechanism as part of a recycling pathway (15). The transferrin receptor is expressed in a ubiquitous manner but is rela-
tively enriched in proliferative cells and cells of the hematopoietic derivation (14). It was demonstrated that gene transfer could also be accomplished through the transferrin internalization pathway (Fig. 1). In addition to these molecular conjugate vector strategies, gene delivery via the receptor-mediated endocytosis pathway has been described utilizing antibody against the polymerized IgA receptor (11) as well as surfactant protein C (18) as the ligand domain of the vector. Thus, the receptor-mediated schema of gene delivery has been developed for an increasing number of gene transfer applications to capitalize on the potential to achieve nontoxic cellular entry as well as cell-specific gene delivery.

Despite the many potential advantages of receptor-mediated gene delivery, the efficacy of molecular conjugate vectors, in practice, has been idiosyncratic (4,8). For many target cells, despite the presence of the requisite cell surface receptor, gene transfer via the corresponding internalization pathway has not resulted in efficient expression of transferred DNA. Analysis of the cellular uptake of conjugate-DNA complexes in these instances has frequently demonstrated effective cellular internalization of the conjugate-DNA complex (3). In this regard, the internalized complexes may be demonstrated within cellular endosomes. This finding suggests that the inefficient gene expression is not related to initial binding and internalization steps, but reflects events occurring after cellular entry of the DNA-conjugate complex. Thus, in some instances, the conjugate-DNA complex may be entrapped within cellular endosomes and the DNA thus cannot access the nucleus. Consistent with this concept, it has been reported that treatment of cells with selected lysosomotropic agents can significantly augment gene expression in conjugate-transfected cells (8). This is consistent with the fact that loss of conjugate-DNA complexes through cellular degradative processes may be etiologic of their inability to achieve nuclear localization and thus expression of transferred genes. Taken together, these findings illustrate a fundamental flaw of receptor-mediated gene transfer strategy as initially conceptualized: Despite the fact that the molecular conjugate vector possesses an efficient mechanism to achieve cellular internalization, the fact that it lacks a specific mechanism to escape entrapment within the endosome limits effective gene transfer and expression. This suggests that if the molecular conjugate vector possessed an endosome escape mechanism, the internalized DNA could avoid targeting to cellular pathways eventuating its destruction, with a favorable outcome on gene transfer efficiency.

1.1. Adenovirus Facilitation of Receptor-Mediated Gene Delivery

In developing methods to overcome endosome entrapment of the conjugate-DNA complexes, consideration was given to the entry mechanism of certain viruses. For both enveloped and nonenveloped viruses, the first step to cellular
entry involves binding to a specific cellular receptor. For a subset of these viruses, after binding, internalization occurs by virtue of the receptor-mediated endocytosis pathway (19,20). After cellular internalization, these viruses then utilize specific mechanisms to allow endosome escape of their genetic material so that the viral life cycle may be completed in the cell nucleus or cytosol. The viral entry pathway thus possesses certain parallels to the entry pathway of molecular conjugate vectors; like the conjugate vector, viruses may possess specific and efficient receptor-mediated entry mechanisms. However, apart from the conjugate vector, after internalization, viruses possess specific mechanisms to achieve escape from entrapment within the cell vesicle system for completion of their infectious cycle. It was thus hypothesized that the endosome escape property of certain viruses could potentially be exploited to overcome endosome entrapment of the conjugate-DNA complex, and thus facilitate efficient gene transfer. In this regard, previous studies had linked viral entry to enhanced cellular uptake of various macromolecules. Carresco utilized both enveloped and nonenveloped viruses to show augmented cellular internalization of macromolecules (21). These studies did not delineate the mechanistic basis for this phenomenon, or distinguish between membrane-bound or fluid-phase molecules; however, the linkage between viral uptake and enhanced cellular entry of heterologous molecules was clearly established. Specific facilitation of ligands via the receptor-mediated pathway was demonstrated by Pastan et al. (22). This group has developed a system of antitumor therapeutics based on delivery of ligand-toxin chimeras via receptor-mediated endocytosis. In these studies, it was found that entrapment of the chimeric toxin molecule in the cellular endosome limited tumoricidal efficacy. It was noted in this instance that this limitation could be overcome by codelivery of adenovirus with the chimeric toxin. In this schema, the virus colocalized within the same cellular endosome as the conjugate during internalization. Further, it could be shown that it was the adenovirus' ability to disrupt cellular endosomes that allowed ingress of the conjugate into the cytosol, where its activity was thus potentiated. Thus, this work established that adenovirus enters cells via receptor-mediated endocytosis, and during this process, it may colocalize to cellular endosomes with other receptor-bound ligands. The virus exits the endosome via a membrane disruption step that may also allow egress of other endosome contents.

From the standpoint of exploiting this capacity of the adenovirus to achieve endosome escape, it is noteworthy that this effect is mediated by viral capsid proteins and independent of viral gene expression. In this regard, the entry cycle of the adenovirus has been partially elucidated (19,23,24). The virus first achieves target cell attachment through binding to a specific cell surface receptor. Whereas this receptor has not been characterized, its presence has been
established by functional studies and it has been shown to be expressed in a rather ubiquitous manner. The adenovirus accomplishes this initial binding step by virtue of a specific capsid protein designed fiber. After binding to the cellular recognition receptor, uptake is facilitated by subsequent binding of specific viral capsid regions to cellular proteins that act as uptake “triggers” (25). After this initial uptake step, the virion is then localized within cellular endosomes. After cellular localization within the endosome, the virus accomplishes endosome disruption to achieve escape from the cell vesicle system. Acidification of the endosome is crucial to the ability of the virus to achieve this vesicle disruption step. In this regard, cellular targets with defective acidification of their endosomes, or alternatively, treatment of normal cells with agents to augment endosomal pH, both have the effect of limiting adenoviral propagation (26). Importantly, replication-defective adenoviral strains deleted in specific gene regions may still, nonetheless, complete the same principal entry steps as wild-type viruses. It is hypothesized that acidification of the endosome induces alterations in the conformation of certain capsid proteins (19,26). This induces changes in their hydrophobicity allowing them to thus interact with the endosome membrane in a manner to achieve vesicle disruption. Thus, it is the capsid proteins that mediate the effect of endosome disruption, viral gene expression is not an essential feature of this process.

Based on this concept, it was hypothesized that the entry process of the adenovirus could be exploited to achieve endosome escape of the internalized conjugate-DNA complex. In this schema, the provision of endosome disruption functions in trans would be anticipated to augment the overall gene transfer efficiency mediated by the molecular conjugates (Fig. 2). To test this hypothesis, transferrin-polylysine-DNA complexes containing a firefly luciferase reporter plasmid DNA were codelivered to HeLa cells in conjunction with the replication-defective adenovirus dl312. This use of the replication-defective adenovirus provided a convenient means to separate the possible effects mediated by viral entry and viral gene expression, as this adenovirus strain in defective in its gene expression secondary to genomic deletions in early viral gene regions (27). It could be seen that with increasing input of adenovirus there was a corresponding increase in the level of luciferase gene expression detected (9). This adenoviral augmentation plateaued at a level of luciferase gene expression more than 2000-fold the levels observed when transferrin-polylysine conjugates were utilized without virus. Significantly, the amount of virus required to achieve these plateau levels of augmented gene expression corresponded to the number of receptors for the adenovirus on the target cell (28). Thus, the characteristics of receptor-mediated endocytosis facilitation by this route were saturable, as would be expected in a receptor-limiting context.
Fig. 2. Mechanism of adenoviral facilitation of molecular conjugate-mediated gene transfer. After binding to their respective cell surface receptors, cointernalization of the transferrin-polylysine conjugate and the adenovirus is within the same endocytotic vesicle. Adenovirus-mediated endosome disruption allows vesicle escape for both the virion and the conjugate-DNA complex.

To determine the mechanistic basis of the virus' ability to augment gene transfer mediated by molecular conjugates, steps were employed to uncouple virus entry and virus-mediated endosome disruption. In this regard, mild heat treatment of the adenovirus will selectively ablate the ability of the adenovirus to accomplish endosome disruption, without impairing its ability to bind to target cells (28). Additionally, this magnitude of heat treatment does not affect the structural integrity of the adenoviral genome. Thus, by selectively ablating the viral endosome disruption capacity, its contribution to adenoviral-mediated augmentation of molecular conjugate gene transfer could be ascertained. In these experiments, heat treatment completely abrogated the ability of the virus to augment the conjugate's gene transfer capacity. This finding establishes that it is specifically the viral property of endosomolysis that contributes to its capacity to augment the conjugate’s gene transfer capacity. This underscores the fact that it is the molecular conjugate vector’s lack of an endosome escape mechanism that represents its principal limitation to achieving efficient gene transfer to target cells.
The augmented levels of net gene expression accomplished by the adenoviral augmentation of molecular conjugates are consistent with either increased gene expression in a transfected cell subset or an increased number of cells transfected. To distinguish between these possibilities, HeLa cells were transfected with transferrin-polylysine conjugates containing a β-galactosidase reporter gene (29). Cells transduced with the transferrin-polylysine-DNA complexes alone demonstrated a transduction frequency of <1%. When adenovirus was added as a facilitator, however, >90% of the HeLa cells showed expression of the β-galactosidase gene. Thus, the adenoviral augmentation of conjugate-mediated transfer allowed for a significantly enhanced frequency of transfection. To demonstrate further the level to which adenovirus could increase the efficiency of gene transfer mediated by conjugate-DNA complexes, limiting dilutions of transferrin-polylysine-luciferase DNA complexes were delivered to cells with or without adenovirus. It could be demonstrated that, in the presence of adenovirus, the same levels of heterologous gene expression were noted as when two orders of magnitude more DNA were delivered without adenoviral augmentation (9). Thus, the virus appears to confer a high level of efficiency on the process of DNA delivery mediated by molecular conjugates. Importantly, the phenomenon of adenoviral augmentation of conjugate-mediated delivery could be observed in a variety of cell types treated in this manner. In analyzing multiple different cellular targets, the free adenovirus significantly augmented gene expression levels over levels seen with molecular conjugates alone. Additionally, certain cell types that appear refractory to transferrin-polylysine-mediated gene transfer demonstrate susceptibility only in the presence of adenovirus (9). It is likely that, in these instances, there was effective internalization of conjugate-DNA complex, but heterologous gene expression was absent secondary to more complete endosome entrapment of the conjugate-DNA complex. Thus, the susceptibility of these cells to conjugate-mediated gene transfer was only manifest after codelivery of adenovirus.

The selective exploitation of adenoviral entry features is made possible because it is adenoviral capsid proteins that mediate the endosome disruption step of viral entry. In this context, viral gene expression is irrelevant to the ability of the adenovirus to facilitate molecular conjugate entry. Steps may thus be undertaken to ablate viral gene elements and spare the capacity of the capsid to accomplish cell vesicle disruption. In this regard, it has been shown that ultraviolet (UV) light and UV light plus psoralen can be used to ablate viral infectivity and allow retention of the virus’ ability to facilitate molecular conjugate-mediated gene transfer (29). Thus, in this context, it is possible to render the adenoviral genome inactive in two complementing manners: genetic deletions of the adenoviral genome and physical inactivation of the adenoviral
DNA-Adenovirus Conjugates

This is in marked contrast to recombinant viral vectors, whereby the integrity of viral gene elements is crucial, since the heterologous sequences are contained therein. Thus, for recombinant viral vectors, it is not possible to take steps such as UV treatment to more completely inactivate viral gene elements. An additional feature that derives from this strategy is the flexibility allowed in DNA delivery. In this regard, the polylysine component of the molecular conjugate interacts with DNA in a sequence-independent manner. Thus, DNA of any design can be incorporated into the conjugate-DNA complex and delivered for purposes of gene transfer. Furthermore, the fact that the heterologous sequences are not incorporated into a viral genome minimizes the possibility of interactions among the distinct regulatory regions. Of additional practical significance, because the heterologous DNA is not packaged into a virion capsid, the amount of DNA that may thus be delivered is not limited by the corresponding packaging constraints. Using this approach, DNA plasmids of up to 48 kb have been delivered (29). Thus, an enhanced flexibility in terms of size and design of delivered DNA derives from this strategy of gene transfer.

1.2. Adenovirus-Component Molecular Conjugates for Receptor-Mediated Gene Delivery

The utility of the adenovirus in facilitating adenoviral entry in trans suggests that it might also be possible to accomplish this with the adenovirus functioning in cis. Thus, since molecular conjugates were functionally limited by their lack of an endosome escape mechanism, and since adenovirus possessed such a mechanism, it seemed logical to incorporate the adenovirus into the structure of the molecular conjugate vector. The first technical barrier to achieving this construction was the attachment of the adenovirus to the polylysine-DNA binding moiety. In attaching moieties to the adenoviral capsid, a potential complication undermining this strategy would have been perturbation of the capsid proteins involved in adenoviral binding and entry. In this regard, the adenoviral capsid proteins fiber and penton are thought to be of major importance to these entry steps (19,26). The hexon protein is thought to subserve the function of “scaffolding” of the capsid and is less important in viral entry processes. It was thus determined that the most propitious site to accomplish linkage was via the hexon protein. To achieve this, the strategy delineated in Fig. 3 was carried out. The hexon gene of the adenovirus was first isolated. Specific mutations were introduced into the gene sequence of the hexon gene to create a region coding for a heterologous epitope. The gene sequence altered corresponded to a region of the hexon protein known to be present in the exterior, surface face of the virus. Thus, by genetic techniques, it was possible to generate a chimeric adenovirus with the heterologous epitope
Fig. 3. Construction of chimeric adenovirus containing heterologous epitope in surface region of hexon capsid protein. Since the adenoviral capsid proteins fiber and penton are important mediators of the adenoviral entry mechanism, attachment of capsid-bound DNA was targeted to the hexon protein. A specific attachment site for an immunologic linkage was created by introducing a heterologous epitope into the surface region of the hexon protein by site-directed mutagenesis of the corresponding region of the adenoviral hexon gene. The introduced foreign epitope is a portion of Mycoplasma pneumoniae P1 protein.

localized in a manner to permit nonneutralizing interaction with an MAb specific for this epitope. After derivation of the chimeric adenovirus, an attachment schema could be carried out (Fig. 4). The antibody specific for the heterologous epitope served as the site of attachment of the polylysine-DNA-binding moiety. When condensed with DNA, the resulting toroid possessed surface localized immunoglobin capable of recognizing the heterologous epitope on the chimeric virus.

The ability of the adenovirus-polylysine-DNA complexes to mediate gene transfer was evaluated using various components of the complete complex. It could be seen that the specific combination of epitope-marked virus, antibody-polylysine, and DNA resulted in a vector that was capable of achieving high levels of heterologous gene expression (13). In contrast, any other combination of these components did not allow gene transfer to occur. As before, heat inactivation of the virion ablated the capacity of the complexes to accomplish gene transfer, indicating that it was the viral entry features that were responsible for the gene transfer capacity of the complex in this context as for the case of adenovirus functioning in trans. As an additional control, complexes were
Fig. 4. Schematic of approach to derive adenovirus-polylysine-DNA complexes containing heterologous DNA attached to exterior of adenovirus capsid. To accomplish linkage of adenovirus and a polycationic DNA-binding domain, the chimeric adenovirus containing a heterologous epitope in the exterior domain of its hexon protein was employed in conjunction with an MAb specific for this epitope. Control experiments demonstrated that attachment of the MAb was nonneutralizing for the adenovirus. The MAb was rendered competent to carry foreign DNA sequences by attaching a polylysine moiety. Interaction of the polylysine-antibody complexed DNA with the epitope-tagged adenovirus occurs via the specificity of the conjugated antibody. Constructed with polylysine-condensed DNA that lacked linker antibody. Again, no gene transfer was noted. This emphasizes that the physical linkage between the virus and the DNA is the crucial feature of the complex that allows effective gene transfer to occur. To determine the net efficiency of the complexes in mediating gene transfer, logarithmic dilutions of formed adenovirus-polylysine-DNA complexes were made and applied to target HeLa cells. It could be seen that as few as 10 DNA molecules per cell resulted in levels of reporter gene expression detectable above background levels (13). This compares favorably to gene transfer mediated by other DNA-mediated gene transfer methods, where on the order of 500,000 DNA molecules per cell are required (30,31). Thus, the adenovirus-polylysine-DNA complexes are capable of extremely efficient gene transfer.
For linkage of the polylysine-DNA-binding moiety to the adenoviral exterior, specific steps were undertaken to avoid perturbation of adenoviral capsid proteins relevant to endosome disruption. It has also been shown that these specific steps are not obligatory for functional incorporation of the adenovirus into the configuration of the molecular conjugate vector Wagner et al. have shown that DNA-polylysine complexes may be attached to the adenoviral capsid by enzymatic means, employing transglutaminase and by exploiting the biotin-streptavidin interaction, using streptavidin-polylysine conjugates in conjunction with biotin-labeled adenovirus (6). It has also been shown by Christiano et al. that direct chemical linkage of adenovirus and polylysine may be achieved utilizing the heterofunctional crosslinking agent EDC (32). Adenovirus-component molecular conjugates derived by these methods possess activity profiles comparable to complexes employing immunologic methods of attachment.

When compared to the maximum levels of gene transfer achieved by free viral facilitation of molecular conjugate-mediated gene transfer, adenovirus in the linked configuration is capable of significantly higher levels of gene expression (data not shown). This likely reflects the different entry pathways of delivered DNA in the two different contexts. In this regard, for free adenovirus facilitation of molecular conjugates, the virus enhances gene transfer by disrupting cell vesicles to allow ingress of the conjugate-DNA complex into the cytosol. In this schema, the complex does not possess any specific mechanism to achieve localization in the nucleus after cell entry. In contrast, in the linked configuration, after endosome disruption, the complex would possess the additional capacity to localize to the nuclear pore, based on the adenoviral moiety’s capacity to accomplish this localization (33). In this location, the overall dynamics of gene transfer are likely more favorable for accomplishing expression of the heterologous genes.

In the adenovirus-polylysine-DNA complex configuration, the adenovirus moiety was incorporated to function in the capacity of an endosome disruption agent. In this vector design, however, it also represents the unique ligand domain of the molecular conjugate. Thus, it would be expected that the relative gene transfer efficiency of this vector would be determined by the relative tropism of the adenovirus for a given cellular target. This is indeed the case, as cells with a relatively high number of surface receptors for adenovirus, HeLa, and KB, are highly susceptible to gene transfer by this vector system. In contrast, cells with relatively fewer cell surface receptors for adenovirus, such as HBE1 and MRC-5, have a correspondingly lower susceptibility to gene transfer by the adenovirus-polylysine-DNA complexes (13). To overcome this potential limitation, strategies were developed to determine if the adenovirus could be employed as an endosomolysis agent in conjunction with an alterna-
DNA-Adenovirus Conjugates

Fig. 5. Strategy for the employment of combination complexes containing adenovirus and transferrin. Complexes were derived that contain transferrin as the ligand domain and adenovirus as an endosomolysis domain. These combination complexes possess the potential to enter cells via the transferrin or adenovirus pathway. In the former instance, after entry via the transferrin pathway, the adenovirus would function in the capacity of an endosomolysis agent. Such conjugates thus possess both specific internalization and endosome escape mechanisms.

tive ligand. This strategy would presumably overcome the limitation of adenoviral receptor targeting of the adenovirus-polylysine-DNA complexes. The derivation of the combination complexes is shown in Fig. 5. This vector construction involves the sequential addition of adenovirus, linker antibody-polylysine, DNA, followed by a second ligand-polylysine. The result would be a complex containing multiple independent functional domains: a ligand domain to target specific cell subsets, an endosomolysis domain to enhance overall gene transfer efficiency by accomplishing cell vesicle escape, and a DNA binding domain.

The combination complexes were delivered to HeLa cells, a cellular target that has receptors for both adenovirus and the alternative ligand, in this case transferrin. It was observed that the combination complexes mediated even further augmentation of gene expression (13). This augmented level of gene expression is clearly of greater magnitude than an additive effect of transferrin-polylysine and adenovirus-polylysine would predict. This suggests that, effectively, there is some element of cooperativity related to the ability of the
complexes to enter cells for DNA delivery. This would appear to be because these complexes are capable of entering cells by either the transferrin or adenovirus pathway, and after entry, are capable of achieving cell vesicle escape. To establish this concept, the combination complexes were delivered to target cells relatively lacking adenovirus receptors. The HBE1 cell line was demonstrated to be refractory to transduction with adenovirus-polylysine-DNA complexes based on an absence of adenoviral receptors. When transduced with the combination complexes, however, levels of gene expression comparable to those seen in HeLa cells were noted. In this instance, the complexes achieve entry by nonadenoviral pathways, thus demonstrating the utility of providing a second ligand in the complex design.

With the recognition that high efficiency gene delivery could be achieved by including an endosomolytic domain into the design of the molecular conjugate, alternate strategies were developed to incorporate this functional attribute. In this regard, there are ample rationales for attempting to eliminate intact adenovirus from the conjugate configuration. First, the incorporated virion possesses the capacity to function as an alternate ligand domain, potentially undermining the desired property of cell-specific targeting. In addition, despite the ability to inactivate the viral adenoviral genome, its presence represents a potential safety concern. Thus, attempts have been made to isolate the portion of the adenoviral capsid that mediates cell vesicle disruption for functional inclusion into the molecular conjugate. These attempts, however, have been unsuccessful, likely reflecting the fact that adenovirus-mediated cell vesicle disruption is a complicated process, requiring the coordinated actions of multiple capsid components (personal communication; E. Wagner) This recognition also negatively bears on attempts to utilize recombinant capsid components deriving from adenovirus. As an alternative, Cotten et al. have recently employed a xenotropic adenovirus for this purpose. The adenoviral strain CELO was able to perform as an endosomolysis agent at functional levels comparable to human serotypes (34). The disadvantage of this strategy derives from the relative difficulties encountered in preparing large amounts of this reagent. From the biosafety standpoint, the optimal endosome disruption agent would be a synthetic product to circumvent any issues associated with virus or viral products. Wagner et al. have explored the utility of synthetic viral peptides with fusogenic properties (35). Whereas infusion of these peptides into the molecular conjugate structure augment gene transfer efficiencies, the levels of augmentation observed are far inferior to those observed with adenovirus. Thus, for the present, the adenovirus provides the most efficacious means of conferring on molecular conjugates the capacity to achieve cell vesicle escape and thus accomplish high-efficiency gene delivery.
1.3. Strategies to Enhance Capacity for Cell-Specific Receptor-Mediated Gene Delivery

In the combination complexes, multiple independent domains provide multiple independent functions. These functions operate in a concerted manner to facilitate overall gene transfer. The incorporation of the adenovirus provides the important functional attribute of cell vesicle escape for the complex. However, by virtue of its binding capacity through its fiber protein, it provides an alternative ligand domain for the complex. This alternative source of binding potentially undermines the capacity to achieve cell-specific targeting, which is one of the theoretical attributes of the molecular conjugate vector system. Thus, it would be desirable to exploit the endosomolytic capacity of the adenovirus without allowing it to behave as an alternative ligand. One strategy to achieve this end is to block adenoviral binding with an antibody specific for the cell receptor binding domain of the fiber protein (36). This type of antibody was derived by immunization with purified adenoviral fiber protein and screening for antibodies with neutralizing capacity. Thus, the capacity of the antibody to block adenoviral binding and internalization was first established. Next, it was determined whether the antibody blockade of adenoviral entry could ablate the ability of free adenovirus to facilitate molecular conjugate-mediated gene transfer. In this analysis, it could be seen that the antifiber antibody ablated the ability of the adenovirus to facilitate molecular conjugate-mediate gene transfer. This confirms that cellular entry of the virus is crucial to its capacity to augment conjugate entry. The next step was to derive combination complexes whereby the adenovirus had been precoated with antibody against the fiber protein to block entry via viral receptor pathways. It was hypothesized that the complex could nonetheless enter via the pathway of the alternative ligand, transferrin, whereby the attachment-blocked virus would mediate endosomolysis. In this analysis, the utilization of binding-incompetent virus as a component of the complex did not decrease the overall levels of gene transfer observed. This indicated that fiber binding was not required for the virus to mediate endosome disruption. Indeed, the process of adenoviral binding and endosome disruption are not functionally linked. Thus, it is possible to construct a molecular conjugate vector whereby the adenovirus is incorporated exclusively in the capacity of an endosomolytic agent and does not function as a competition ligand.

Ablation of the adenovirus moiety as a potential competitor ligand enhances the overall specificity of the conjugate vector for a given target cell. Besides the adenovirus, however, there exist additional sources of nonspecific binding within the conjugate design. For example, it has been shown that certain cells bind to polylysine-DNA-complexes in a nonspecific manner (16). In these instances, when nonligand-polylysine-DNA complexes are codelivered to cells
with adenovirus, they may accomplish levels of gene transfer comparable to ligand-polylysine complexes. This is, therefore, another potential confounding factor undermining the potential to achieve a vector system possessing cell-specific binding capacity. Since this nonspecific association between polylysine and the target cell surface is on an electrostatic basis, it was hypothesized that steps could be employed to neutralize the charge differential and thus ablate the basis of this nonspecific attachment. To this end, yeast tRNA, a negatively charged polynucleotide, was employed to treat polylysine-condensed DNA. It could be seen that the treatment significantly reduced the nonspecific binding mediated by the polylysine-DNA complex (37). To determine whether the tRNA interfered with specific ligand-polylysine internalization, transferrin-polylysine-DNA complexes were treated in a similar manner and analyzed for their capacity to achieve binding and internalization when codelivered with adenovirus. In this study it could be seen that the tRNA did not interfere with specific ligand-polylysine gene transfer. Thus, this maneuver is capable of reducing nonspecific cell binding of the polylysine component of the ligand-polylysine complexes without affecting the specific receptor-mediated endocytosis uptake of ligand-polylysine complexes. This offers the potential to enhance the cell specificity of the conjugate vector.

Thus, various maneuvers to eliminate potential sources of nonspecificity in the molecular conjugate design may be employed. These strategies may be employed in the various ligand-based strategies to achieve cell-specific gene delivery. In this regard, the specificity of the molecular conjugate is determined by its ligand domain, which dictates the cellular binding of the vector. Employed ligands may be physiologic ligands, as in the case of asialoglycoprotein and transferrin. In addition, the ligand may represent a synthetic mimic of a physiologic ligand. This has been accomplished for targeting the asialoglycoprotein receptor by Wagner et al. as an asialoglycoprotein mimic (35). Antireceptor antibodies have also been used as in the studies of Curiel et al. where anti-Ig immunoglobulin served as ligand for cell surface immunoglobulins for targeting B-lymphocytes (38). The use of viral proteins with specific affinity for cell-type markers has also been demonstrated by targeting CD4 human lymphocytes with HIV gp120-ligand conjugates. In these instances, ligands were employed to direct molecular conjugate vectors into pathways that were known a priori to be associated with efficient cellular internalization. It was hypothesized that it might also be possible to successfully target conjugates employing ligands that do not bind to characterized cellular internalization pathways. In this regard, Batra et al. have developed molecular conjugates exploiting the utility of cell surface-lectin interactions for vector targeting. It was found that lectins could successfully facilitate effective gene delivery mediated by molecular conjugates (39). Presumably, in this instance,
cell surface binding of the lectin-polylysine-DNA complex is sufficient to induce a level of internalization permissive of effective gene delivery. Thus, targeting of molecular conjugate vectors may be designed to exploit characterized cellular receptor-mediated endocytosis pathways. Additionally, in some instances, the employment of ligand moieties possessing cell binding affinity may be sufficient to allow functionally relevant internalization of the conjugate-DNA complex.

The design of adenovirus-component molecular conjugate vectors represents a unique manner of exploiting viral entry features to achieve gene delivery. This strategy is thus distinct from the design of recombinant viral vectors. In the instance of recombinant viral vectors, the overall entry mechanism of the virus is exploited to achieve gene delivery through the incorporation of the foreign gene into the genome of the virus. For the adenovirus-component molecular conjugate vectors, viral entry features are exploited to facilitate gene transfer in a highly selective manner. This possibility arises from the fact that the capacity of the adenovirus to achieve entry is an intrinsic property of its capsid proteins. This mechanism may thus be incorporated into a vector system whereby the viral gene elements are obviated. The vector design that derives from this strategy results in a system with potential advantages related to utility and safety. Furthermore, the development of a vector system with the engineered capacity to achieve cell-specific gene delivery is in conceptual accord with the concept of a “targetable-injectable” vector (40). The further development of the capacities within the present vector design may allow the achievement of a vector system possessing many of the attributes of this proposed vector ideal.

2. Materials

1. Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin is used for routine cultivation of tissue culture cells. For particular experiments, this is modified as indicated in Section 3.
2. CsCl 1.33: 454.2 mg/mL CsCl in 5 mM HEPES, pH 7.3.
3. CsCl 1.45: 609.0 mg/mL CsCl in 5 mM HEPES, pH 7.3.
4. HBS: HEPES 5 mM, pH 7.3, 150 mM NaCl.
5. Poly-L-lysine. Poly-L-lysine (Sigma, St Louis, MO) 500-mg bottle dissolved in 50 mL HBS and pH adjusted to 7.3. The final volume is brought up to 84 mL with HBS and reagent aliquoted in 500-μL volumes with storage at −20°C.
6. EDC: (Pierce, Rockford, IL) 1 g EDC is dissolved in 4.0 mL dH₂O at time of use and stored at 4°C. This must be discarded immediately after use. Unused EDC powder should be stored under argon at −20°C.
7. Viral preservation medium: Combine the following: 1 mL 1 M Tris-HCl (pH 8.0), 2 mL 5 M NaCl, and 0.1 g bovine serum albumin (BSA). Bring volume up to 50 mL and mix until BSA in solution. Add 50 mL glycerol, filter sterilize, and store at 4°C.
3. Methods

3.1. Large-Scale Viral Preparation of Adenovirus

1. Grow 293 or HeLa or WI-62 to ~80% confluence in 75-cm flasks (~10^7 cells/flask) x 20 in DMEM/10% FCS/PCN + Strep.
2. Aspirate off media
3. Dilute viral stock in 100 mL DMEM/2% heat inactivated FCS (2% FCS media) and add 5 mL/flask. Added viral stock should be approx 10^10–10^11 viral particles to achieve MOI ~100–1000/cell.
4. Incubate 37°C, 5% CO2, 1–2 h.
5. Add 10 mL complete media/flask (DMEM/10% FCS/PCN + Strep)
6. Incubate as above 48–72 h until prominent cytopathic effect is seen in cells.
7. Harvest cells and media from flasks in 50-mL capped centrifuge tubes.
8. Centrifuge 1000 rpm, 10 min, 4°C Beckman GS-GR centrifuge or other tabletop centrifuge.
9. Aspirate off supernatant and resuspend combined cell pellets in total volume of 20 mL 2% FCS media
10. Freeze-thaw 4X (may use 37°C bath alternating with dry ice/ethanol bath)
11. Centrifuge as in step 8 except at 4000 rpm, 20 min, 4°C, and harvest supernatant; save at 4°C while setting up gradients.
12. Set up CsCl gradient tubes as follows:
   a. SW 28 tube. Step 20 mL 1.33 CsCl (discontinuous gradient)
   b. 10 mL 1.45 CsCl (discontinuous gradient).
   c. Overlayer gradient with 5 mL viral cell lysate.
   d. Centrifuge 18,000 rpm, 1.5 h, 20°C.
13. Harvest lowest opalescent band needle puncture in tube with aspiration into syringe (in approx 2 mL).
14. Dilute 1:1 with 5 mM HEPES, pH 7.3.
15. SW41 tube.
   a. 4 mL 1.33 CsCl (discontinuous gradient).
   b. 4 mL 1.45 CsCl (discontinuous gradient); overlayer with 4 mL virus diluted from 1st spin.
   c. Centrifuge 25,000 rpm, overnight (>18 h), 20°C.
16. Harvest lower band as before in minimal volume.
17. Use virus directly for chemical coupling to poly-L-lysine or preserve as follows: dilute: 1 part virus to 5 parts viral preservation media
18. Store in aliquots at -70°C.
19. To measure virus concentration: Mix 100 μL of adenovirus solution with 900 μL 1X TE and determine A260 by spectrophotometry. One A260 equals approx 10^12 viral particles per mL

3.2. Chemical Linkage of Adenovirus and Poly-L-Lysine

1. Virus is prepared as in Section 3.1.; after the 2nd spin, resuspend virus in 2.5 mL final volume with 1.33 CsCl.
2. Load onto a PD10 column (Pharmacia, Uppsala, Sweden) pre-equilibrated with HBS
3. Elute with 2 mL HBS.
4. Resuspend to a final volume of 3.6 mL with HBS
5. Reaction: Mix 3.6 mL virus, 0.4 mL poly-L-lysine, and 0.04 mL EDC. Leave on ice for 4 h.
6. Mix well with 8 mL of 1.45 CsCl.
7. Centrifuge at 25,000g for 18 h in an SW41 tube.
8. Harvest the opalescent band—dilute with viral preservation medium to achieve concentration of $1 \times 10^{11}$ particles/mL, and aliquot. Store at $-70^\circ$C

### 3.3. Preparation of Adenovirus-Polylysine-DNA Complexes

Recipe for 6.0 μg DNA (transfection of $\sim 1 \times 10^6$ cells); may be scaled up or down accordingly.

1. 100 μL Adenovirus-polylysine (EDC-linked AdpL@ $1 \times 10^{11}$ particles/mL).
2. 6.0 μg plasmid DNA in 200 μL HBS (20 mM HEPES, 150 mM NaCl, pH 7.3), 30 min RT.
3. 4.0 μL poly-L-lysine (pL) in 200 μL HBS, 30 min, RT. Or, 6.0 μL human transferrin-polylysine (Serva, 1 mg/mL) in 200 μL HBS, 30 min, RT.
4. Add to cells that are in 1/2 total vol 2% fetal calf serum media, 60 min., 37°C.
5. Add 1/2 vol of media with fetal calf serum (FCS) to bring final FCS concentration to maintenance level.

### 4. Notes

For certain applications, adenovirus-component molecular conjugate vectors are ideal in their present form. For example, they have been found to be highly efficient transfection reagents for many in vitro applications. In addition to mediating high-efficiency gene transfer in transformed human cells, they are able to mediate high-efficiency gene transfer in primary cell cultures of various tissue types. This is of significance in ex vivo transduction of cells that are to be retransplanted after transfection with the gene of interest. Another important property of the molecular conjugate design is its ability to deliver large DNA constructs, which is a great limitation in recombinant viral systems. This allows the use of specific promoter elements to enhance overall gene expression. In addition to delivering large genes efficiently, it is also possible to deliver multiple DNA constructs simultaneously.

Despite their efficacy in vitro, use of molecular conjugates in vivo has been idiosyncratic. Gao et al. have shown that molecular conjugate vectors are able to mediate gene transfer in the airway epithelium of cotton rats (41). The observed gene transfer was not, however, as efficient as would be expected from the observed in vitro efficacy. This has been shown to be owing to the instability of molecular conjugates in vivo. It has been shown that the poly-
lysin component of the conjugates is a target of humoral factors after in vivo delivery (E. Wagner, personal communication). Since the basis of conjugate instability is understood, several steps may potentially be taken to address this problem. As the polylysine component is the primary locus of conjugate instability in vivo, it is logical to propose the replacement of the polylysine DNA-binding moiety with some other DNA-binding component to obviate this limitation. An alternate strategy would be to directly link the DNA to the ligand via chemical techniques using crosslinking reagents or with a high affinity biotin-streptavidin linkage. In addition, a conceptually distinct strategy being developed seeks to mask the polycation component of conjugates using stealthing procedures. These are several examples of steps that may be taken to improve the in vivo efficiency of molecular conjugate vectors by mitigating the known basis of conjugate instability. All of these strategies are presently in the development stage.

In their present form, molecular conjugate vectors are able to mediate high efficiency gene transfer via the receptor-mediated endocytosis pathway and thus possess the capacity for targeted delivery. This is largely owing to the plasticity of the conjugate design whereby incorporated ligands determine the vector tropism. It has been shown that viral endosome disruption functions incorporated into the conjugate design dramatically enhance gene transfer efficiency, and that these functions may be exploited selectively in a manner whereby the viral tropism will not undermine conjugate specificity. In addition, since the endosome lysis ability of the adenovirus is not a function of viral gene expression, it is possible to selectively exploit this function without introducing viable viral gene elements, thus taking steps to inactivate the viral genome. This strategy seeks to capitalize on the minimal functional elements of the virus entry pathway that are useful for vector utility. Though not ideal in its present form, this vector design is a developmental step toward the concept of a targetable, injectable gene transfer vector. It is flexible in its tropism and is largely comprised of specifically derived functional components. The next logical step in vector development would be the incorporation of an integration mechanism into the design of the vector. This would enable the delivered DNA to be stably maintained in the host genome leading to long-term gene expression. As it has been demonstrated that distinct functional domains may operate in an independent manner in the context of molecular conjugate vectors, it is not illogical to speculate that a viral integration system could also be incorporated into the design of the present system.

Acknowledgments

The author wishes to express his gratitude to his coworkers Frosty Loechel and Sharon Michael at the University of Alabama at Birmingham; Raj Batra and
Ling Gao at the University of North Carolina, Chapel Hill; and Matt Cotten and Ernst Wagner at the Institute of Molecular Pathology in Vienna, Austria.

References


Methods for the Construction and Propagation of Recombinant Adeno-Associated Virus Vectors

Jeffrey S. Bartlett and Richard J. Samulski

1. Introduction

The development of gene transfer vectors from the human parvovirus, adeno-associated virus (AAV), has provided scientists with an efficient and effective way of delivering genes into mammalian cells. This chapter aims to explore the various practical aspects of the AAV vector system, and in consequence, to highlight particular difficulties that may be encountered by workers new to the field. However, before describing the methodology involved in the generation of recombinant AAV vectors, it is of value to briefly discuss the structure and life cycle of this unique virus. Detailed and more extensive reviews that describe the biology of adeno-associated virus are also available (1–3).

1.1. AAV Structure and Genetics

The AAV genome is encapsidated as a single-stranded DNA molecule of plus or minus polarity (4–7). Strands of both polarities are packaged, but in separate virus particles (4), and both strands are infectious (8). The genome of AAV-2 is 4675 bp in length (9) and is flanked by inverted terminal repeat sequences of 145 bp each (10,11) (Fig. 1). The first 125 nucleotides of these terminal sequences are palindromic and fold back on themselves to form T-shaped hairpin structures that are used to initiate viral DNA replication (for details see reviews by Berns et al. [12,13]). The terminal repeats also contain the sequences necessary to package the viral DNA into virions (14–16). Studies of AAV replication, latent viral chromosomes, and defective interfering particles all point to the viral terminal repeats as the key cis-acting elements required for a productive AAV infection.
1.2. AAV Life-Cycle and the Development of AAV Vectors

Adeno-associated virus is a defective member of the parvovirus family. The AAV can be propagated as a lytic virus or maintained as a provirus that is integrated into the host cell genome (2). In a lytic infection, replication requires coinfection with either adenovirus (17–19), or herpes simplex virus (20); hence the classification of AAV as a defective virus. The requirement of a helper virus for a productive infection has made understanding the AAV life cycle more difficult. However, from a vector point of view, it has added a level of control when generating nonreplicative vectors, in that they can be propagated under controlled conditions (see Section 3.1.), thereby reducing unwanted spread and providing an important margin of safety. One of the most interesting aspects of the AAV life cycle is the virus' ability to integrate into the host genome. When AAV infects cells in the absence of helper virus, it establishes latency by persisting in the host cell genome as an integrated provirus (3,21,22).
Although AAV establishes a latent infection, if these cells are super-infected with wild-type helper virus, the integrated AAV can be rescued from the chromosome, and re-enter the lytic cycle.

As a prerequisite for vector construction, it was necessary to gain an understanding of the AAV life cycle. This problem was approached by first cloning a double-stranded version of the virus into a plasmid backbone (23,24). Since wild-type AAV could be rescued from an integrated chromosome and enter the lytic cycle following adenovirus infection, it was of interest to determine if such a recombinant AAV plasmid could be used for generating wild-type AAV virus. It was determined that the viral genome could be rescued from the plasmid recombinant backbone by transfection of the plasmid DNA into human cells in conjunction with adenovirus type-5 as helper virus. Similar infectious plasmid clones have been the template for all subsequent vector constructions. The ability to generate large quantities of plasmid DNA that is basically inert until introduced into adenovirus-infected human cells also provides a safe and efficient way of manipulating this system.

The first use of AAV as a vector for the transduction of a foreign gene into the host chromosome was demonstrated by Hermonat and Muzyczka in 1984 (14). A recombinant AAV (rAAV) viral stock was produced using an infectious plasmid vector similar to that described above in which the neomycin resistance gene (neo') was substituted for the AAV capsid genes. This rAAV was able to transduce neomycin resistance to both murine and human cell lines (14).

Since these first studies, AAV has been used as a viral vector system to express a variety of genes in a number of different eukaryotic cells (11,25–28). All of these experiments have used plasmid vectors in which portions of the AAV genome were substituted with the foreign gene of interest. The size of the inserted non-AAV or foreign DNA is limited to that which permits packaging of the rAAV vector into virions (4.7 kb), and thus depends on the size of the retained AAV sequences.

1.3. Minimal AAV Vectors

Although the early studies described in Section 1.2. demonstrated the potential use of AAV as a vector, several technical problems remained: the need for efficient packaging systems, methods for producing recombinant virus stock free of wild-type AAV, and the identification of minimum AAV sequences required for transduction. This last hurdle would have direct impact on the size of foreign DNA inserts. In attempts to solve these problems, constructs that retained only a limited number of nucleotides from the viral terminal sequences were tested (8,15,16). The remainder of this discussion will focus on the AAV plasmid construct psub201 (8), which is a derivative of the original recombinant viral vector described in Section 1.2. (24). This plasmid vector contains
Fig. 2. Plasmid vectors for the generation of recombinant AAV. The present method for producing stocks of rAAV utilizes a two-component plasmid system; AAV plasmid vector, i.e., psb201, and AAV helper plasmid, i.e., pAAV/Ad. Foreign DNA is inserted between the two XbaI sites of psb201, removing the Rep and Cap regions of the AAV genome, are supplied in trans by the plasmid pAAV/Ad.

two XbaI cleavage sites flanking the viral coding domain (8), such that the entire viral coding domain can be excised and foreign DNA sequences can be inserted between the cis-acting terminal repeats (Fig. 2). Vectors derived from this plasmid have been shown to transduce genes at frequencies similar to the earlier vectors, suggesting that the minimal cis-acting sequences needed in the viral plasmid are only the AAV terminal repeats. Recently, similar vectors have been developed by others. These plasmids are all able to carry foreign gene cassettes of 4.6–5.0 kb in size.

1.4. rAAV Packaging Systems

The present method for producing stocks of recombinant AAV utilizes a two-component plasmid system: AAV plasmid vector, as described in Sections 1.2. and 1.3.; and AAV helper plasmid, which provides the necessary AAV capsid and replication proteins in trans. An important consideration is that the vector and helper plasmid DNAs should be sufficiently nonhomologous so as to preclude homologous recombination events between the two, which could generate wild-type AAV. Although there are many variations on this theme, this discussion will focus on the AAV helper plasmid, pAAV/Ad, which contains adenovirus type-5 terminal sequences in place of the normal AAV termini. This plasmid has no homology to the AAV vector plasmid and cannot be packaged into AAV virions since it lacks the terminal cis-acting domains required for this function. In addition, this hybrid plasmid does not contain adenovirus packaging sequences, thereby eliminating the potential for unwanted adenovirus recombinants.
By cotransfecting the helper plasmid \( pAAV/Ad \) and the vector \( psub201 \), containing a foreign gene inserted between the two AAV terminal repeats (in the presence of adenovirus), rescue, replication, and packaging of the foreign gene into AAV particles occurs. The adenovirus genome has been shown to activate the adenovirus terminal repeats on \( pAAV/Ad \); this enhances the turning on of the AAV genes. The \( rep \) gene products recognize the AAV \( cis \)-acting terminal repeats on the vector \( psub201 \) containing a foreign gene, rescue these recombinant molecules out of the plasmid, and begin to replicate them. The AAV capsids begin to accumulate, and they recognize the AAV \( cis \)-acting packaging signals located in the AAV terminal repeats and encapsidate the recombinant viral DNA into an AAV virion. The result of such a packaging scheme is an adenovirus helper and AAV particle carrying the recombinant DNA. The adenovirus helper can be removed by a number of physical and genetic techniques. Heating the virus lysate to 56°C for 30 min is one such strategy. In this packaging system, one can generate helper-free stocks of AAV vectors at titers of \( 10^4-10^5/mL \).

### 1.6. Advantages and Disadvantages of rAAV Vectors for Gene Transfer and Gene Therapy

As mentioned, several AAV vector systems have been developed that consist of a recombinant plasmid capable of being packaged into AAV particles. To date, about 96% of the AAV genome can be replaced with foreign DNA and packaged into an AAV virion. In using this strategy, one starts with an infectious plasmid that effectively removes all of the coding capacity of the AAV genome. The \( cis \)-acting AAV terminal repeats that are retained do not appear to contain dominant enhancer or promoter activity, and recombinant viruses generated using these elements function as vectors for stable transduction. Expression of the gene or DNA sequence of choice in eukaryotic cells is determined by the control of a transcriptional promoter included with the gene cassette (29,30).

Recombinant AAV is among the newest of the possible genetic transfer vectors. This once obscure virus possesses unique properties that distinguish it from all other vectors. Its advantages include the ability to integrate into the mammalian genome and the lack of any known pathogenicity. Its ability to carry regulatory elements (i.e., tissue-specific enhancers/promoters, splice sites, and so on) without interference from the viral genome allows for greater control of transferred gene expression. In vitro experiments demonstrate that rAAV vectors can transduce primary hematopoietic cells, and they support the development of this vector system for gene transfer (31,32).

Disadvantages currently exist from the inferior packaging systems that yield low numbers of recombinant virions that are contaminated with wild-type
adenovirus. The present packaging systems are very inefficient. Yields of wild-type AAV usually exceed $10^{10}$ virus/mL, whereas recombinant AAV titers are in the $10^4$–$10^5$ virus/mL range. Thus far, two approaches have been reported for removing adenovirus from AAV stocks: heat inactivation and CsCl density centrifugation. The latter method is also an effective way of concentrating AAV virus stocks. Additionally, it may be possible to use an adenovirus with a temperature-sensitive mutation (particularly one in the adenovirus DNA polymerase or structural genes) to produce an AAV vector stock that would be essentially free of adenovirus contamination. Fortunately, these difficulties do not seem to be insurmountable technical problems. Finally, the limitation of the small genome able to be packaged (~5 kb) suggests that genes or cDNAs averaging 3–5 kb will be the most appropriate to be used in this vector.

2. Materials

2.1. Cell Culture—General

1. Human 293 cells. The cells are split 1/6 every 3–4 d. They should not be allowed to overgrow and should be low passage (<p50). The human 293 cells can be obtained from American Type Culture Collection, Rockville, MD (ATCC cat no CRL 1573).

2. Phosphate-buffered saline (PBS). 1.0% NaCl, 0.025% KCl, 0.14% Na$_2$HPO$_4$, 0.025% KH$_2$PO$_4$ (all w/v), pH 7.2; sterile

3. Dulbecco’s Modified Eagle’s Medium (DMEM) with glutamine containing 100 U/mL penicillin G and 100 µg/mL streptomycin

4. Tissue culture dishes (100-mm diameter)

5. Fetal bovine serum (FBS)

6. Phosphate-buffered saline containing 0.5 mM EDTA (PE).

2.2. Recombinant AAV Plasmid Vector

The use of the AAV vector psub201 requires that the foreign gene cassette be excised with a restriction enzyme that produces $XbaI$ sticky ends. Alternatively, the insert and digested vector must be treated with a suitable enzyme to produce flush ends for blunt-end ligation. The inserted gene must have appropriate promoter and maybe enhancer sequences, contain sufficient protein coding regions, have appropriate polyadenylation signals, and should contain an intron for maximal expression with some promoters. Such manipulations and the construction of similar gene cassettes are described elsewhere (33). Once the foreign gene has been inserted into the AAV vector, it may be advisable to sequence across both ends of the gene to ensure against mutation.

It is important to use very pure DNA for transfection. Plasmids should be prepared and purified by double CsCl gradient centrifugation (see Note 1).
2.3. Helper Plasmid

The rationale behind the construction of AAV helper plasmids such as pAAV/Ad has been discussed in Section 1.4. Very pure DNA should be used for transfection. Therefore, helper plasmid should also be purified by double CsCl gradient centrifugation (see Notes 1 and 2).

2.4. Generation of Recombinant Virus

1. Monolayers of 293 cells at approx 80% confluency
2. Lipofectin reagent (Gibco-BRL), or Transfection-reagent (DOTAP) (Boehringer Mannheim, Mannheim, Germany).
3. Polystyrene tubes (Falcon, Los Angeles, CA): 17 x 100 mm (#2059) or 12 x 175 mm (#2058) (see Note 7).
4. Tissue culture dishes (see Section 2.1).
5. DNA samples: CsCl-purified preparations of both recombinant AAV plasmid and helper plasmid (see Section 2.1).
6. Opti-MEM medium (Gibco-BRL).
7. Dulbecco’s Modified Eagle’s Medium (DMEM) (as described in Section 2.1.), containing 10% FBS.
8. Phosphate-buffered saline (PBS; see Section 2.1).
9. HEPES-buffered saline (HBS): 20 mmol/L HEPES (N-2-Hydroxyethyl-piperazine-\(\text{N}^\prime\)-2-ethanesulfonic acid), 150 mmol/L NaCl, final pH 7.4. Filter sterilized and stored at 5°C
10. PE solution (see Section 2.1.)
11. Adenovirus stock (type dl309).

2.5. Isolation and Purification of Recombinant Virus

1. Phosphate-buffered saline (PBS; see Section 2.1.).
2. Clinical centrifuge for low speed centrifugation.
3. Sonicator (for example, Branson Model 2000, fitted with 0.5-cm microtip).
4. TNE Buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8.0).
5. Dounce homogenizer
6. Dialysis tubing.
7. 2% Trypsin solution.
8. 20% Sodium deoxycholate solution.
9. Solid CsCl.
10. CsCl solutions for density gradient centrifugation at 1.7 g/mL, 1.5 g/mL, 1.35 g/mL, and 1.25 g/mL in 0.01M Tris-HCl, pH 8.0; 0.001M EDTA.
11. Ultracentrifuge capable of 150,000g.

2.5. Titration of Recombinant Virus

1. Ninety-six-well tissue culture dishes.
2. DMEM containing 10% FBS, and DMEM containing 2% FBS (see Section 2.1).
3 Adenovirus stock (type dl309) at known titer (see Section 2.1).
4. Wild-type AAV stock at predetermined titer
5. PE solution (see Section 2.1).
6. Nylon hybridization membranes, 47-mm circular.
7. Phosphate-buffered saline (see Section 2.1).
8. Millipore 47-mm filter holder, or equivalent.
9. 0.5N NaOH solution
10. 1.0M Tris-HCl, pH 7.5.
11. Solutions for standard DNA hybridization analysis, including the preparation and purification of radioactively labeled DNA probes
12. X-ray film (Kodak X-OMAT)

3. Methods

3.1. Generation of Recombinant Virus

This section outlines methods for cotransfecting 293 cells with two plasmids, for rescue of the recombinant AAV genome and packaging into viral particles. The cell line of choice is always 293, because these cells are good recipients of DNA in DNA-mediated gene-transfer procedures, and allow the use of replication-deficient adenovirus as helper virus.

We routinely use one of the transfection procedures outlined below to obtain infectious recombinant virus from recombinant plasmid clones. Although we have investigated a number of other methods (calcium-phosphate precipitation, DEAE-dextran, electroporation, and so on), we have found that the method of choice is most often the one the investigator has the most experience with and is most comfortable with. The reader is encouraged to evaluate other transfection techniques as well.

3.1.1. Transfection of DNA Using Lipofectin Reagent

1. Grow 293 cells on 100-mm tissue culture dishes in DMEM containing 10% fetal bovine serum, until they are 60–80% confluent.
2. Suspend DNAs in 1.0 mL Opti-MEM medium per 100-mm tissue culture dish in a 15-mL polystyrene tube. The amount of DNA used for each transfection is related to the amount of Lipofectin reagent and the number of cells (see Note 8).
   We routinely use 5–25 µg of total DNA; pAAV/Ad helper plasmid, and rAAV vector plasmid at a 3:1 ratio, per 100-mm tissue culture dish.
3. Suspend Lipofectin reagent in another 1.0 mL of Opti-MEM medium in a second polystyrene tube. The DNA/Lipofectin ratio should be 1:4 (see Note 8).
4. Mix the DNA and Lipofectin together by inverting the tube several times or vortexing gently.
5. Incubate the DNA/Lipofectin mixture for 5 min at room temperature.
6. Aspirate the medium from the cells and wash the monolayer twice with either Opti-MEM medium or PBS.
7. Add the DNA/Lipofectin/Opti-MEM mixture (2 mL) to the cells
8. Incubate at 37°C under normal 5% CO₂ conditions for 3–6 h.
9. Aspirate the media and replace with DMEM containing 2% FBS and adenovirus at 3–10 IU/cell.
10. Continue incubation for 48 h.

3.1.2. Transfection of DNA Using DOTAP Transfection Reagent

1. Grow cells on 100-mm tissue culture dishes in DMEM containing 10% serum, until they are 60–80% confluent.
2. Dilute DNAs (see Section 3.1.1. and Note 8) to 250 µL with HBS in a 15-mL polystyrene tube.
3. Dilute DOTAP transfection reagent to 250 µL with HBS in a second polystyrene tube. The DNA/DOTAP ratio should also be about 1:4 (see Note 8).
4. Mix the DNA and DOTAP solutions together and incubate for 10 min at room temperature.
5. Add Opti-MEM medium to bring the total volume to 5 mL and mix gently.
6. Aspirate the medium from the cells and wash the monolayer twice with either Opti-MEM medium or PBS.
7. Add the DNA/DOTAP/Opti-MEM mixture (5 mL) to the cells
8. Incubate at 37°C under normal 5% CO₂ conditions for 6–24 h.
9. Aspirate the media and replace with DMEM containing 2% FCS and adenovirus at 3–10 IU/cell.
10. Continue incubation for 48 h.

3.2. Isolation and Purification of Recombinant Virus

3.2.1. Rapid Crude Virus Stocks

Concentrated crude virus stocks are prepared from infected 293 cells by scraping the cells infected as described in Section 3.1. into the culture medium and recovering them by pelleting at low speed in a bench-top centrifuge. The cells are then resuspended in a hypotonic buffer, e.g., PBS, and the viruses are released by either three cycles of freeze-thawing or brief sonication. The cell debris can be removed by a second low speed centrifugation if desired, and the lysate stored at -70°C until needed.

Prior to using this crude cell lysate to infect cells, it may be desirable to inactivate the adenovirus by heating the cell lysate at 56°C for 1 h. This treatment will effectively remove the heat-labile adenovirus from the preparation without decreasing the titer of recombinant AAV significantly.

3.2.2. Purified Stocks

Although the rapid, crude viral stocks, prepared as described in Section 3.2.1., are often all that is needed to produce infectious recombinant AAV, in vivo gene transfer necessitates the production of large, high titer, viral stocks.
Since the titer of recombinant AAV from the two-component plasmid system described here is relatively low, the following procedure may be scaled up as needed. Although the volumes described here are per 100-mm tissue culture plate, 150-mm plates or larger vessels may also be used. For larger tissue culture dishes, some amounts may need to be adjusted in proportion to the change in culture dish surface area. This protocol should facilitate the recovery and concentration of rAAV from large volumes of culture media as may be needed.

1. At 48 h postinfection, scrape the cells into the media and collect by low speed centrifugation (500g, 5 min). Remove the media from the cell pellet and resuspend the cells in 10 mL of TNE buffer.

2. Add 1.0 mL 0.2% trypsin solution and 10 mL 20% deoxycholate. Mix gently by inverting the tube several times and incubate at 37°C for 1 h.

3. Dounce homogenize the sample 20 times to shear the cellular DNA.

4. Add 5.0 g of solid cesium chloride, mix well to dissolve, and divide the sample between two, 14 x 89 mm, Ultra-Clear (Beckman, Fullerton, CA) ultracentrifuge tubes. Centrifuge at 150,000g in a Beckman SW-41 rotor (or equivalent) for 20 h.

5. After centrifugation, the recombinant viral band may be visible. However, it should not be confused with the slightly less dense adenovirus-helper. If visible, collect the banded virus with a syringe through the side of the tube. If there is no discrete rAAV band, the gradient should be fractionated. Ten 0.5-mL fractions are convenient. Recombinant AAV should band at a density of 1.42–1.45. Determine the density of CsCl in each fraction by either weighing a small amount of each fraction or by using a refractometer. Pool those fractions with densities in the appropriate range. If the rAAV being produced can be easily assayed for biologically (i.e., β-galactosidase or luciferase enzyme assay), this activity may also be used to determine which fractions contain recombinant virus.

6. Dialyze the banded virus in boiled dialysis tubing for about 2 h against two changes of 100 vol of PBS to remove the cesium from the preparation.

7. Layer the dialyzed banded virus on the following CsCl step gradient in a Beckman SW-41 centrifuge tube, or equivalent: 0.5 mL CsCl at 1.7 g/mL, 1.5 mL CsCl at 1.5 g/mL, 3.0 mL CsCl at 1.35 g/mL, 1.5 mL CsCl at 1.25 g/mL.

8. Mark the outside of the centrifuge tube at each step interface with a permanent marker.

9. Centrifuge at 150,000g in an SW-41 (Beckman) rotor for 2 h.

10. Recombinant AAV should again band at a density of 1.42–1.45. The band will probably not be visible, but will form at the interface between the 1.35 and 1.5 CsCl steps. Collect the banded virus with a syringe through the side of the tube.

11. Dialyze the banded virus in boiled dialysis tubing for about 2 h against two changes of 100 vol of PBS to remove the cesium from the preparation.

12. Freeze aliquots at −70°C. These virus stocks are stable for many years at −70°C.
3.3. Titration of Recombinant AAV by Replication Center Assay

It is often important to determine the titer of recombinant AAV. Since AAV is a defective virus, direct titration of infectious units by their ability to form plaques on permissive cells is not possible. Therefore, rAAV must be indirectly titered by a replication center assay (as described here), or by its ability to transduce an assayable marker gene. Since the replication center assay will be more applicable to a broader range of rAAV vectors, it is described here.

1. The day prior to performing the assay, plate 293 cells into 96-well tissue culture dishes. The cell density should be approx 2-3 x 10^4 cells/well (about 50% of confluence). Set up one row of the plate for determining the recombinant titer (row A), and a second row for wild-type AAV contamination (row H). Up to 11 different preparations of AAV can be titered on a single plate (see Fig 3).

2. The next day, remove the media from the cells, leaving behind just enough to cover the cells. Add 1.0 µL of the rAAV-containing lysate, or purified rAAV preparation, to a well in the first row on the plate (row A), and the same amount to a well in the last row (row H). Other wells can be used to monitor rAAV purification or concentration as described in Section 3.2. Leave the last well in the row A for an “Ad only” control. Rock the plate gently back and forth to mix.

3. In a 15-mL polystyrene tube, add a predetermined amount (see step 4) of adenovirus to 2.4 mL of DMEM/2% FBS. Mix well. Dispense 100 µL per each well in row H, and the Ad only control well at the end of row A.

4. To the remaining, approx 1.2 mL of the media/Ad mixture, add a calculated amount of wild-type AAV and dispense 100 µL per each well in row A. The final MOI of adenovirus and AAV should be 20 and 2, respectively.

5. Incubate for 30 h.

6. Remove the media and rinse the cells with a small volume of PBS.

7. Add 250 µL of PE solution to each well and place the dish on ice.

8. Mark each nylon hybridization membrane with pen, prewet with PBS, and mount in the filter holder.

9. Apply 5.0 mL of PBS to each filter.

10. Resuspend the cells from a well of the 96-well plate by pipeting up and down. Apply 50 µL of the cell suspension into the PBS buffer on the top the nylon filter mounted in the filter holder; mix gently. Apply suction.

11. Carefully remove the nylon filter disk from the manifold with flat-tipped forceps. Place the disk with cell side up onto a pool of 0.5N NaOH on plastic wrap. Stretch the plastic wrap to ensure that all disks wet evenly. After 2 min, remove disks and place on blotting paper (cell side up).

12. Repeat step 11 with a new sheet of plastic wrap and fresh 0.5N NaOH.

13. On a new sheet of plastic wrap, pipet a small amount of 1.0M Tris-HCl, pH 7.5. Place the nylon filter disks with cell side up onto the pool of 1.0M Tris-HCl, pH 7.5. Stretch the plastic wrap to ensure that all of the disks wet evenly. After 2 min, remove the disk and place on blotting paper (cell side up).

14. Repeat step 13 with a new sheet of plastic wrap and fresh 1.0M Tris-HCl, pH 7.5.
Fig. 3. Infectious center assay. Cells are grown in a 96-well plate, as described in the text, resuspended in PBS and applied to nylon filter membranes. After denaturation, and binding of the replicated AAV DNA to the filters; the filters are probed with radioactively labeled wild-type, or foreign gene, DNA probe to determine the titer of the recombinant AAV preparation (see text for details).

15. Allow the disks to dry at room temperature. Fix the DNA to the disks by microwaving for 5 min at full power.
16. Hybridize the filters from row H with a radioactively labeled wild-type AAV probe and from row A with a radioactively labeled foreign gene DNA probe.
Hybridization using probes with a specific activity of $10^7$ cpm/µg DNA gives a strong signal on overnight exposure of the filter to X-ray film.

17. The titer of the original rAAV preparation is equal to the number of positive cells per filter multiplied by $5 \times 10^3$, expressed as infectious units (IU) per milliliter.

4. Notes

1. All DNA preparations should be pure; double banding through cesium chloride gradients (34) or a preparation of equivalent high quality is essential.
2. The AAV terminal repeats may be unstable in plasmids propagated in several commonly used laboratory strains of *E. coli*. Host strains carrying *recA*, as well as *recJ* and *recB* mutations, such as the SURE (Stratagene, La Jolla, CA) strain of cells, are recommended.
3. The small (30-nm diameter) cationic lipid vesicles in the Lipofectin and DOTAP reagents can fuse to form large (>1-µm diameter) multilamellar structures in the presence of polyvalent anions, such as EDTA, citrate, or phosphate (35). Preparations of these large, fused vesicles are less efficacious than the small vesicles. Therefore, tissue culture media containing high concentrations of polyanionic buffers should be avoided.
4. There are components present in serum that can inhibit Lipofectin-mediated transfection. Although a wide variety of serum-free media give good results with lipofection, in a number of cell lines, Opti-MEM I (Gibco-BRL) gives the best results with respect to both viability in the presence of Lipofectin and the level of expression obtained. DOTAP transfection reagent is less inhibited by the presence of serum; however, we still recommend the use of serum-free media.
5. The toxicity of the transfection reagent varies among different cell types, and elimination of serum from the transfection media further reduces viability. Screening of serum-free media has identified Opti-MEM as an acceptable serum-free medium, which results in improved viability, relative to DMEM, for human 293 cells.
6. Large nonfunctional DNA/lipid aggregates can form when the DNA concentration during formation of the Lipofectin/DNA complexes is too high (approx 100 µg/mL or greater). Formation of these large aggregates is exacerbated by increased ionic strength; if it is desirable to prepare a concentrated stock solution of premixed Lipofectin/DNA, or DOTAP/DNA, complexes, they should be prepared at low ionic strength. In an effort to maintain both the DNA and transfection reagent concentrations at the lowest possible concentration, prior to complex formation, the DNA and transfection reagent should be separately diluted into equal volumes of media and then mixed.
7. The concentration-dependent, ionic species and ionic strength-dependent aggregates that can form under different transfection conditions are sticky, and they can be seen to adhere to glassware and to plastic. Polypropylene and glass attract these aggregates more than polystyrene. For this reason, polystyrene mixing containers are preferred for transfection.
Optimum transfection activity occurs under conditions in which the net negative charge on the DNA is substantially reduced. Complexes with an excess of positive charge are taken up by cells more effectively than neutral or negatively charged complexes. The optimum transfection activity for DNA seems to occur when the ratio of positively charged molar equivalents (contributed by DOTMA in Lipofectin, or DOTAP in the Transfection-Reagent from Boehringer Mannheim) exceeds by 1.0–2.5 the number of molar equivalents of negative charge contributed by the DNA. The molarity of DOTMA (669.5 mol wt) in a 1 mg/mL Lipofectin solution that contains 50/50 (w/w) of DOPE (neutral lipid) is 0.75 mM; the molarity of DOTAP (774.2 mol wt) in a 1 mg/mL solution is 1.3 mM, and the molar equivalents of negative charge in a 1 mg/mL DNA solution (average mol wt of the nucleotide monomer is 330) is 3 mM. Based on this estimate, the optimum activity occurs when the total mass of lipid (DOTMA + DOPE, or DOTAP) exceeds the mass of DNA by 4–10-fold.

Since unsaturated fatty acids are oxidized by airborne oxygen, it is recommended that the DOTAP transfection reagent be removed from the bottle with a sterile cannula and syringe. This will avoid a subsequent reduction of transfection efficiency.

As compared to DOTMA, DOTAP has the advantage that it can be decomposed by nonspecific esterases in the cells after fusion and deposition, respectively, although this may have limited relevance to the production of rAAV.

Virus yields will be dependent on plasmid transfection efficiency, titer of adenovirus helper, level of AAV gene product expression, the length of time post-infection prior to harvesting, and the ability to remove and concentrate virus from the culture medium. The need to optimize as many variables as possible is apparent.

When making virus stocks, it is important not to grow the virus in multiple passages; this will lead to the generation of defective particles that will decrease the efficiency of expression of inserted genes.

References

Recombinant Adeno-Associated Vectors


Generation of High-Titer, Helper-Free Retroviruses by Transient Transfection

Warren S. Pear, Martin L. Scott, and Garry P. Nolan

1. Introduction

Retroviral gene transfer is presently one of the most powerful techniques for introducing stably heritable genetic material into mammalian cells (reviewed in ref. 1). One serious drawback of this technique, however, has been the difficulty in readily producing high-titer recombinant retroviruses. For many applications, such as infecting rare target cells or the majority of cells in tissue culture, the recombinant virus titer must be at least $10^6$ infectious units/mL. Although one can usually obtain high-titer mixtures of recombinant and replication-competent retroviruses in a relatively short time, many applications such as cell marking studies or studying genes in vivo demand freedom from replication-competent virus.

A milestone in gene transfer technology was the creation of cell lines that could package retroviral RNAs into infectious particles without the concomitant production of replication competent virus (2,3) (reviewed in refs. 4 and 5). In what became the prototype for creating helper-free retrovirus packaging cells, the retroviral structural gene products (gag, pol, and env) were provided in trans by separating them from the elements required for packaging retroviral RNA into virions. The first ecotropic retroviral packaging line, ψ2, was created by stably introducing into NIH3T3 cells an engineered retroviral DNA genome from which the RNA packaging signal, termed ψ, had been removed (2). Production of high-titer, helper-free, infectious virus required the subsequent stable introduction of a retroviral vector containing the ψ retroviral packaging site, transcription and processing elements, and the gene of interest into the ψ2 cells (see Chapter 4). Retroviral particles produced in this way could infect target cells and transmit the gene of interest, but not replicate, because
genomic information encoding the packaging proteins was not carried in the packaged retroviral particles. This original strategy has subsequently been modified by a number of investigators to decrease the risk of producing replication-competent virus and to increase viral titers (see reviews in refs. 4 and 5).

Although infectious retrovirus is produced within 48 h following transfection of these packaging lines, the infectious titer generated by these cells is generally low ($10^3$-$10^4$/mL), necessitating identification of those clones that produce retroviruses at higher titer. This is accomplished by selecting single cell clones from the transfected population, then testing each for its ability to produce virus (Fig. 1A). Under optimal circumstances, this requires 1–2 mo
Retroviruses by Transient Transfection

and must be repeated for each different construct. During the prolonged selection process, gene(s) encoded by the retroviral vector may inhibit growth of the packaging line, favoring the outgrowth of clones that express low levels of virus, thereby making it difficult to identify clones continuously producing retrovirus at a high titer. Although the biochemical basis for this effect is often unclear, the inability to produce high-titer infectious retroviruses expressing certain genes, such as members of the abl and rel families, has been experienced by a number of investigators (7).

To minimize potential toxic effects of the introduced gene products on the packaging cell lines, several groups have devised strategies based on transient retroviral production (6-8). This approach has the added benefit of markedly reducing the time and effort required to produce high-titer retroviral stocks. Although the first step in both stable and transient retroviral production is transfection of the retroviral construct into the packaging cell line, at 48 h after transfection, the infectious retroviral titers are several logs higher using the transient production methods (Fig. 1B).

Several factors may account for the ability of the transient retroviral production methods to yield viral supernatants with infectious titers in the range of $10^5$–$10^7$/mL. These include both the properties of the parent cells themselves and changes engineered into them to augment transient production. Unlike previous retroviral packaging cell lines based on either murine or avian cells, the transient approaches utilize cells derived from either primates or humans. These cells offer several potential advantages. First, they are much more transfectable using simple CaPO₄-based protocols. Second, they support very high level expression of genes introduced under control of a number of promoters, such as those derived from CMV. Third, they contain fewer endogenous retroviral loci whose products could interfere with infectious virus assembly and release (9). In addition, they have been previously modified to contain viral gene products, such as adenoviral E1A or polyoma large T-antigen, with properties that can be exploited to further enhance expression of new constructs.

The strategy described by Landau and Littman (6) uses transient cotransfection of the SV40-transformed African green monkey renal cell line, COS-7, with two modified retroviral plasmids, both containing the SV40 origin of replication. In addition to this viral element, one construct contains the MLV LTR, retroviral packaging site, SV40 origin of replication (ori), and DNA element(s) of interest, whereas the second construct encodes retroviral core (gag), polymerase (pol), and envelope (env) proteins but lacks a retroviral packaging site. Virus stocks with titers as high as $10^5$ infectious units/mL have been obtained with certain recombinant constructs by using this approach. The ability to obtain titers in this range depends, in part, on large T-antigen-dependent
amplification of the plasmids containing the SV40 ori, which can enhance infectious recombinant titers by up to 2 logs (6). Although these authors do not report the detection of replication competent virus, the vectors used in this study could produce such genomes after only one recombination event.

Both of the other transient transfection strategies (7,8) use the 293 cell line, an adenovirus-transformed human embryonic kidney cell line (10) as the basis of their packaging systems. In the approach described by Pear et al. (7), two different plasmids encoding the retroviral structural genes, in trans, were stably incorporated into the 293T cell line, a variant of 293 cells into which a temperature-sensitive SV40 large T-antigen had been introduced (11). As the retroviral structural genes are encoded on two different plasmids and contain additional mutations, at least three recombination events are necessary to generate replication-competent retroviruses (12). Stable incorporation of these plasmids is important for preventing helper virus formation since transient introduction of these plasmids together with a Moloney-based retroviral vector yielded replication-competent virus (7). Using this system, all constructs tried by the authors have produced retroviral titers greater than $10^6$/mL. This includes not only relatively nontoxic genes such as those encoding β-galactosidase (βgal) and G418 resistance, but also members of the abl and rel families of protooncogenes from which we were unable to identify stable high titer producing clones utilizing the stable production approach (7). These viruses have been used to infect rodent tissue culture cells and murine hematopoietic progenitors with no detectable generation of helper virus (7). Although retroviral vectors containing an extended packaging site (13) give approximately a twofold increase in titer, infectious titers greater than $10^6$/mL have been obtained with all of the retroviral vectors tried by the authors including pGD (14), pBABE (15), MFG (16), and pBND (D. Turner and C. Cepko, unpublished, described in ref. 7). We have observed no enhancement of titer using retroviral vectors containing an SV40 ori, despite the fact that a 293 cell variant (293T) (11) was chosen as the starting material for the packaging cell lines.

The system described by Finer et al. (8), termed kat, also utilizes 293 cells. However, unlike the cell lines of Pear et al. (7), the kat system requires cointroduction of both a plasmid encoding the retroviral structural genes and a plasmid encoding the retroviral vector. The constructs utilized in the kat system have been further modified to minimize the creation of replication-competent retroviruses. In general, the titers obtained using kat-produced retroviral supernatants are similar to those found by Pear et al. (7). It is unclear whether further advantage would result from the creation of a packaging cell line by stable introduction of the kat constructs. Another question is whether all MuLV-based retroviral vectors will function in the kat system or whether only the re-engineered kat vectors function well in this system.
In summary, both the methods described by Pear et al. (7) and Finer et al. (8) give rapid production of high-titer, helper-free viruses. These methods are particularly useful in creating retroviral stocks containing genes that are toxic to stable producer lines. Also, the ability to rapidly produce high-titer retroviral stocks offers novel possibilities for using these viruses, for example in the creation of retroviral cDNA libraries.

The remainder of this chapter will focus on the use of the packaging cell lines developed by the authors for rapid production of helper-free retroviruses with ecotropic, amphotropic, and polytropic host ranges. The basic 293 cell transfection protocols are likely to work equally well with the kat system plasmids, or other complementation systems that provide the requisite packaging proteins and genomic material to package. Procedures are described for optimizing transfection conditions and performing infections of adherent and nonadherent cell types. The advantages of these transient retroviral production methods should facilitate and extend the use of retroviral gene transfer technology. In addition to producing helper-free ecotropic and amphotropic retroviruses, methods are presented for rapidly pseudotyping retroviral virions with alternative envelope proteins.

### 1.1. Construction of the Packaging Cell Lines

A detailed description of the 293T/17, Anjou65, and Bosc23 cell lines can be found in ref. 7. Briefly, a clone termed "293T/17" was isolated from the 293T (293tsa1609neo) population (11) that produced retroviral supernatants capable of infecting NIH3T3 cells at titers greater than 10^6/mL following transient transfection with wild-type Moloney virus (pZap) (17) and a β-gal-expressing retroviral vector (pBND8) (D. Turner and C. Cepko, unpublished, described in ref. 7). The gag-pol expressing plasmid, pCripEnv- (12), which contains a mutation in the envelope region, lacks the packaging site, and replaces the 3' LTR with the SV40 poly(A) site, was transfected into 293T/17 cells along with a plasmid conferring hygromycin resistance (18). Individual clones were selected and tested for reverse transcriptase activity (19), and one clone, Anjou65, which had the highest reverse transcriptase activity, was used in subsequent experiments. The ecotropic envelope expressing construct, pCripGag-2 (12), which contains mutations in the gag region, lacks the packaging site, and replaces the 3' LTR with the SV40 poly(A) site, was transfected into Anjou65 cells along with a plasmid expressing the gpt-resistance gene (20). Individual clones were isolated and tested for the ability to produce high-titer, β-gal-expressing retroviruses. One clone, Bartlett 96, produced β-gal retrovirus with a titer in excess of 10^6/mL following transfection with pBND8. Two rounds of limiting dilution subcloning were subsequently performed, giving rise to the Bosc 23 ecotropic packaging line.
We have recently developed an amphotropic counterpart to the Bosc23 cell line, termed Bing (W. Pear, M. Scott, and G. Nolan, unpublished). The construction of this cell line was similar to the Bosc23, except that the amphotropic envelope expressing construct, pCripAMgag (12), was used in place of pCripGag-2. Another difference between the Bing and Bosc cell lines is that the gene encoding hygromycin phosphotransferase resistance was expressed from a retroviral vector in the Bing cell line. As a result, Bing cell supernatants express approximately 100 CFU of hygromycin phosphotransferase resistance per mL of retroviral supernatant. Retroviruses produced by Bing cells are helper-free and able to infect NIH3T3 cells at the same efficiency as supernatants from Bosc cells, as well as infect a wide variety of mammalian cell lines, including human T-cells.

One of the authors has recently developed a second generation packaging system termed ØNX. The major edition is that the gag-pol construct has anires-CD8 surface marker with which one can follow gag-pol expression in living cells on a cell-by-cell basis. This facilitates checking of the line for continued high-level gag-pol expression using CD8 as proxy. Additionally, non-MMULV-based promoters were used to drive the gag-pol and env genes, further minimizing the potential for recombination with vector LTRs. Otherwise, construction of these cell lines was similar to Bosc23 and the details of these newer lines will be published in the near future (Achacoso and Nolan, in preparation). Amphotropic and ecotropic versions of these cell lines were prepared and shown to stably express gag-pol and env proteins over several months. These lines are helper-free and produce retroviral supernatants that have titers similar to Bosc23 and Bing. The methods described in this chapter work well with the ØNX cell lines.

1.2. Rapid Pseudotyping of Moloney Retroviral Virions with Vesicular Stomatitus Virus G-Glycoprotein

We have successfully produced high-titer VSV-G-pseudotyped virions for infection of human and nonhuman cell types. The production of VSV-G to pseudotype retroviral virions, demonstrated by Burns et al. (21), is shown here in an adaptation of the transient retrovirus system using the Anjou cell line. A more detailed description of the use of this pseudotyping approach to infect several different human cell lines, mouse cells, fish cells, and insect cells will be published elsewhere (J. Caldwell, J. Lorens, P. Achacoso, and G. Nolan, unpublished results). The method outlined in this chapter for pseudotyping does not require the selection of a stable producer clone, as required by the procedure of Burns (21), and results in the production of recombinant virus 3 d after transfection of the construct. The VSV-G-pseudotyped viruses produced in this fully transient system can be concentrated and used to infect multiple human and nonhuman cell types. It is expected that this approach will also be useful
for pseudotyping retroviral virions with designer envelope proteins that confer
cell-type specific infection of target cells. Such specificity should be of therapeu-
tic importance in numerous clinical settings.

The main advantage of VSV-G-pseudotyping is the stability imparted on
the retroviral virion by the VSV-G envelope. This stability allows forconcen-
tration of virion stocks by centrifugation (multiple rounds can be employed for
continued concentration) to very high titers. A second advantage is that the
VSV-G target epitope is a widely expressed lipid in higher eukaryotes. It is the
broad expression of this latter target epitope that expands the potential host
range of retrovirus-mediated gene delivery.

2. Materials

All solutions are prepared using double-distilled water. For reagents used in
tissue culture, it is recommended that they be prepared in disposable plastic
labware. When possible, it is best to order reagents that have been tissue-cul-
ture tested by the manufacturer.

1. Growth medium (GM) for 293 cells and derivatives: The following are added
directly to DME to give the indicated final concentrations: 10% heat-inacti-
vated fetal bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin, 2 mM L-glutamine.
2. Freezing medium: 90% heat-inactivated fetal calf serum, 10% DMSO.
3. Chloroquine: 25 mM chloroquine stock solution prepared in either PBS or GM
and filtered through a 0.2-μM filter and stored at −20°C.
4. 2X HBS: 50 mM HEPES, pH 7.05, 10 mM KCl, 12 mM dextrose, 280 mM NaCl,
1.5 mM Na₂HPO₄. The final pH of the solution should be 7.05 ± 0.05. Filter
through a 0.2-μM filter, aliquot, and store at −20°C. Try to avoid multiple
freeze/thaw cycles. To thaw, warm to room temperature and invert or vortex the
tube to achieve uniform mixing. Although it is unclear why this occurs, the abil-
ity of the 2X HBS solution to produce working CaPO₄ precipitates deteriorates
after 6 mo to 1 yr, even when the 2X HBS solution is stored at −20°C.
5. 2M CaCl₂ Prepare a 2M solution and filter through a 0.2-μM filter, aliquot, and
store at −20°C.
6. Standard fibroblast medium (SFM) for growth of NIH3T3 cells: The follow-
ing are added directly to DME to give the indicated final concentrations: 10% heat-
inactivated donor bovine serum, 100 U/mL penicillin, 100 U/mL streptomycin,
2 mM L-glutamine.
7. Polybrene: The stock concentration is 4 mg/mL (dissolved in PBS and subse-
duently filtered through a 0.2-μM filter and stored at either 4 or −20°C).
8. Constructs used in VSV pseudotyping: The cDNA for the VSV-G polypeptide of
vesicular stomatitis virus (gift of J. Rose, Yale) was inserted into the vector
pME18S (gift of T. Kitamura, DNAX) under the transcriptional control of the
SRα promoter element (an SV40/HTLV-1 hybrid promoter). MFG-lacZ is a retro-
viral vector expressing lacZ under the control of the MLV LTR (gift of Richard
Mulligan, Whitehead Institute).
Packaging cell lines: The 293T/17, Anjou65, Bosc23, and Bing cell lines are available for noncommercial use through the American Type Culture Collection (Rockville, MD). In order to obtain the cell line(s), it is first necessary to complete and return a material transfer agreement. The material transfer agreements can be obtained from: Office of the General Counsel, Attn. Teresa L. Solomon, Esq., The Rockefeller University, 1230 York Avenue, New York, NY 10021-6399. Any questions regarding receipt of your transfer agreement should be addressed to this office. The accession numbers for the cell lines are: Bing (CAK8): amphotropic envelope-expressing packaging line: CCRL 11554. Bosc23: ecotropic envelope-expressing packaging line: CCRL 11270. Anjou 65: gag-pol expressing cell line: CCRL 11269. 293T/17: CCRL 11268.

3. Methods (see Note 1)

3.1. Growing and Freezing the Cells

The 293 cells and the packaging lines derived from these cells are carried in 293 growth media (GM, see Section 2.) and grown in a 37°C degree incubator containing 5% CO₂. To split and passage the cell lines (see Note 2):

1. Gently rinse 1X with PBS (without Ca²⁺ or Mg²⁺)
2. Trypsinize (0.05% trypsin/0.53 mM EDTA) until the cells easily detach and can be readily pipeted into a single cell suspension (see Note 3).
3. Trypsinization is quenched with GM prior to subculturing in fresh medium.

3.2. Freezing 293 Cells and Derivatives

To assure viability of the cell line, it is recommended that the cells be frozen prior to confluence (see Note 4).

1. To freeze: Wash, trypsinize, and quench cells as described in Section 3.1
2. Centrifuge the cells at 500g for 5 min.
3. Remove the media and add 1 mL of freezing solution (see Section 2.) per 10⁶ cells.
4. Transfer to a 2-mL cryogenic vial.
5. Place the freezing vial at -70°C overnight and transfer to liquid nitrogen on the following day.

3.3. Thawing 293 Cells and Derivatives (see Note 5)

1. Remove one vial from liquid nitrogen and thaw rapidly at 37°C.
2. Immediately add 1 mL GM to the freezing vial and gently transfer this solution to a 15-mL sterile, conical screw-cap tube.
3. Add 2 mL of GM and gently mix the tube to allow for osmotic equilibration.
4. Add 10 mL of GM, close the tube, invert several times, and spin cells at 500g for 5 min.
5. Remove the supernatant, resuspend cell pellet in GM, and transfer to a 10-cm tissue-culture dish.

3.4. Transfecting the Packaging Cell Lines (see Notes 1 and 6)

Unless otherwise noted, all conditions are described for 60-mm plates (see Note 7).
Retroviruses by Transient Transfection

Fig. 2. Appearance of Bosc 23 cells at the time of transfection. The panels show (A) $1.5 \times 10^6$ cells, (B) $2.2 \times 10^6$ cells, and (C) $3.0 \times 10^6$ cells that had been plated 24 h prior to photography. (B) The optimal density at which the cells are approx 80% confluent and clumping is minimized. The cells in (A) are too sparse. Although the cells in (C) are too dense for routine transfections, in some cases (described in the text), it may be necessary to use this number of cells. Cells were counted and plated in 4 mL GM on 60-mm dishes, 24 h prior to photography. Pictures were taken using a 10X objective lens.

1. Plate $2.5 \times 10^6$ cells/60-mm plate in 4 mL of GM 24 h prior to transfection. The dish should be approx 80% confluent prior to transfection (see Fig. 2, Note 8).
2. Just prior to transfection, change the medium to 4 mL of GM containing 25 μM chloroquine (see Note 9).
3. In an Eppendorf tube, prepare the transfection cocktail by adding 6–10 μg DNA to H2O such that the final volume is 438 μL. Add 62 μL 2M CaCl2 to the
DNA/H₂O (see Section 2.). Add 500 μL 2X HBS (pH 7.05) by bubbling (see Section 2., and Note 10). Immediately (within 1–2 min) add this solution to the cells and gently agitate to ensure uniform mixing. Return the cells to the incubator.

4. Approximately 10 h after adding the chloroquine-containing medium, remove the medium and gently replace with fresh GM. (If chloroquine was not used in step 3, skip to step 5.)

5. Approximately 16–24 h prior to harvesting the retroviral supernatant, remove the medium, and gently add fresh GM (see Note 11).

6. Harvest the retroviral supernatant 48 h posttransfection (see Note 12, Section 3.5.).

3.5. Harvesting the Retroviral Supernatant

1. When the retroviral supernatant is ready for harvesting, gently remove the supernatant and either filter through a 45-μm filter or centrifuge for 5 min at 500g at 4°C to remove living cells. If the retroviral supernatant is to be used within several hours, keep on ice until it is used. Otherwise, the retroviral supernatant may be frozen, resulting in a minimal loss of viral titer (see Note 13).

2. To freeze the retroviral supernatant, either snap-freeze the tube in liquid nitrogen or place the tube in dry ice. The frozen samples are stored at −70°C.

To thaw the frozen samples, warm for a minimal period of time at 37°C. The retroviral supernatant is ready for immediate use in subsequent experiments.

3.6. Infection of Adherent Fibroblasts

1. Approximately 12–18 h prior to infection, plate 5 × 10⁵ NIH3T3 cells in SFM (standard fibroblast medium) on a 100-mm plate.

2. Prepare a 3-mL infection cocktail consisting of retrovirus-containing supernatant (either fresh or thawed), polybrene at a final concentration of 4 μg/mL, and SFM.

3. Remove the SFM from the NIH3T3 cells and add the 3-mL infection cocktail to the cells.

4. Return to the incubator for at least 3 h (see Note 14).

5. Add 7 mL of SFM to the cells.

6. Harvest (stain, neo select, and so on) the infected cells at 48 h postinfection.

3.7. Infection of Nonadherent Cells by Addition of Retroviral Supernatant (see Note 15)

The conditions described are for infecting in 60-mm plates.

1. Prepare an infection cocktail consisting of the medium in which the target cells are grown, retroviral supernatant, and polybrene (2 μg/mL) such that the total volume is 3 mL (see Note 16).

2. Centrifuge exponentially growing target cells at 500g for 5 min. Remove supernatant and resuspend the cells in the infection cocktail at a concentration of 10⁵–10⁶ cells/mL.

3. Twenty-four hours postinfection, centrifuge the cells (500g for 5 min) and resuspend in the appropriate media for normal growth of the target cells (see Note 17). Allow the cells to grow for an additional 24–48 h before drug selection or other assays, such as staining for lacZ activity.
3.8. Infection of Nonadherent Cells by Cocultivation with Retrovirus-Producing Cells (see Note 15)

Conditions are described for 60-mm plates.

1. Transfect the 293 cells or derivative cell lines as described in Section 3.4., steps 1–4.
2. Twenty-four hours posttransfection, prepare a 3-mL infection cocktail consisting of polybrene at a final concentration of 2 μg/mL, 1 mL fresh or freshly thawed retroviral supernatant, and the nonadherent cells at a density of $10^5$–$10^6$ cells/mL in the appropriate media for normal growth of the target cells (see Note 16). Remove the medium from the cells and gently add the infection cocktail to the cells (Add the cocktail to the side of the plate rather than directly to the cells). Return the cells to the incubator.
3. Forty-eight hours posttransfection, gently remove the medium that will contain many nonadherent cells, and transfer to a conical tube. Centrifuge for 5 min at 500g (see Note 18). Remove the supernatant and gently resuspend the cell pellet in a freshly prepared infection cocktail as described in step 2.
4. With extreme care to avoid disruption of the adherent cells, add the infection cocktail (in which the nonadherent cells have been resuspended) to the wall of the plate (rather than directly to the cells). Return the plate to the incubator.
5. Twenty-four hours later (72 h posttransfection), remove the nonadherent cells from the dish by gently pipeting (see Note 19).
6. Centrifuge the cells (5 min at 500g) and resuspend in the appropriate media for normal growth of the target cells. Allow the cells to grow for an additional 24–48 h before drug selection or other assays, such as staining for lacZ activity.

3.9. Rapid Pseudotyping of Moloney Retroviral Virions with Vesicular Stomatitus Virus G-Glycoprotein (see Notes 1 and 20)

1. Eight hours prior to transfection, plate the Anjou cells at $3 \times 10^6$ cells/60-mm plate in a 1:1 mixture of Anjou cell-conditioned media and fresh GM (see Note 21).
2. At eight hours postplating, add chloroquine to 25 μM and mix by gentle agitation of the media in the plate.
3. Cotransfect 5 μg pME-VSV-G and 5 μg MFG-lacZ by the calcium phosphate precipitation method described in Section 3.4., step 3.
4. Ten hours later, remove the media and replace with at least 5 mL fresh GM.
5. Seventy-two hours later, remove the retroviral supernatant that can either be used immediately, frozen, or concentrated (see step 6, Note 22).
6. The viral supernatant can be concentrated with no apparent loss of titer by centrifugation at 25,000 rpm in an SW41 rotor for 3 h or 50,000g at 5°C for 90 min to 2 h.
7. Infect the target cells using 4 μg/mL polybrene as described in Section 3.6., step 2 (see Note 23).

4. Notes

1. The viral supernatants produced by these methods can contain potentially hazardous recombinant constructs. The user of these systems must exercise due caution in the production, use, and storage of recombinant retroviral virions,
especially those with amphotropic and polytropic host ranges. This caution should apply to all genes expressed by the retroviral vectors. Appropriate NIH and other regional guidelines should be followed in the use of these recombinant retrovirus production systems.

2 It is suggested that the cells not be split at densities more dilute than 1:5 in order to maintain the uniformity of the cells in culture and minimize the outgrowth of clonal variants. The cells should not be allowed to become overconfluent, as this leads to the formation of cell clumps in culture that can cause uneven cell distribution after replating and result in less efficient transfection.

Although it may be possible to carry the cells longer with no loss of efficacy, we generally maintain them for approx 20 passages (2 mo) before establishing a fresh stock by thawing a frozen vial of stock cells (see Section 3 3.). Although we initially suggested that the Bosc23 cells should be maintained in gpt selection media (7), we have since found that, by freezing multiple aliquots of the cell lines (see Section 3.2.) and following the aforementioned recommendations, it is possible to maintain the cells in GM without selection.

In the event that the 293-derived packaging cell lines need to be reselected, the following genes have been inserted into the cell lines together with plasmids encoding the antibiotic-resistance genes noted. The suggested antibiotic concentration is given in parentheses next to its name. SV40 large T: G418 (1 mg/mL), gag-pol: hygromycin (400 µg/mL); ecotropic and amphotropic envelopes. mycophenolic acid (50 µg/mL) All antibiotics are added directly to GM except mycophenolic acid, which is added to the gpt selection medium suggested by Mulligan and Berg (22).

3. The 293 cells and their derivatives are much less adherent than NIH3T3 cell lines and easily detach from the tissue culture dishes after approx 30 s of treatment with trypsin at room temperature.

4. It is important to freeze multiple (50–100) vials of each producer cell line after first receiving and expanding them. This will ensure a ready supply of backup vials to allow for uniform virus production over several years.

5 If the cells are to be carried in selective media, this should not be applied until after the first passage

6. It is recommended that during initial set-up, the user optimize the system by using a retroviral vector expressing an easily assayable marker such as lacZ or a cell surface protein. During optimization, one should check for transfection frequency of the producer clone and test infection rate of target cells. Tests for transfection and infection frequencies using a βgal-based system can be readily measured by βgal staining or FACS staining for βgal activity (see ref. 7 for methods describing the latter procedures) Only when one is satisfied with the transfection conditions and infection rates should one proceed to using vectors with no readily assayable marker.

7. It should be possible to scale up the protocols.

8. The initial plating of the cells may be the most important step in successfully obtaining high retroviral titers. It is extremely important that the cells are not
overly clumped and are at the correct density. Unlike NIH3T3-derived cell lines
the 293-derived packaging cell lines do not readily form well-spread monolayer
ers. Instead, they tend to clump before confluence, and if the clumping is exces-
sive, the cells will never reach confluence during the 48–72-h period following
transfection. In order to prevent clumping, it is essential that the cells be
extremely healthy prior to plating. If they are overconfluent, it may be necessary
to split them 1:2 or 1:3 for several passages prior to plating for transfection. In
addition, the cells are much less adherent than murine fibroblasts and should be
handled very gently when washing and changing medium.

For consistency, it is important to count the cells rather than estimating the
split. The above cell number is optimized for MFG-lacZ. Expression of other
inserts may be detrimental to the growth of the cells. This effect may be noted by
failure of the packaging cell line to reach confluence by 48–72 h posttransfection.
If this occurs, it may be necessary to plate more cells prior to transfection. For
example, with constructs expressing either fas or P210bcr/abl, it is necessary to
plate $3 \times 10^6$ cells/60-mm plate 24 h prior to transfection. In general, the cells
should be plated at a density so that they are 95–100% confluent at 24 h
posttransfection (for an additional discussion of this issue, see Notes 10 and 12).

9. The addition of chloroquine to the medium appears to increase retroviral titer by
approximately twofold. This effect is presumably owing to the lysosomal neu-
tralizing activity of the chloroquine (23). It is extremely important that the length
of chloroquine treatment does not exceed 12 h. Longer periods of chloroquine
treatment have a toxic effect on the cells causing a decrease in retroviral titer.
The range for chloroquine treatment is 7–12 h, with 9–10 h of treatment giving
the best results. For purposes in which achieving maximal retroviral titer is not
necessary, such as when comparing the relative titers of different constructs, it
may be preferable to omit chloroquine treatment. If chloroquine is not used, it is
unnecessary to change the medium prior to transfection. On some occasions,
we have obtained slightly improved transfection efficiencies by adding the
chloroquine to a 1:1 mixture of 293-conditioned media (obtained from any of the
293-based cell lines) and fresh GM.

10. It is important that the pH of the HEPES be adjusted to 7.05 (within 0.05 U).
Although we generally add the HBS to the DNA/CaCl$_2$ solution by bubbling,
equivalent results can be obtained by adding the HBS to the DNA/CaCl$_2$ solution
and immediately inverting the tube. The HBS/DNA/CaCl$_2$ solution should be
added to the cells within 1–2 min of preparation. It is not only unnecessary to
wait for the formation of a visible precipitate, but waiting this long (15–30 min)
may have a detrimental effect on transfection efficiency and subsequent retroviral
titers. In addition, the presence and/or amount of precipitate that one visualizes
following transfection is not a reliable indicator of transfection success.

We have used DNA prepared by both cesium chloride gradients and several
commercial kits and have not found significant differences among titers between
the different preparation methods. It is unnecessary to perform additional phenol
or precipitation steps prior to using the DNA (which is stored in TE [8.0] at $-20^\circ$C).
Up to a point, transfection efficiency and retroviral titers increase with increasing amounts of input DNA. The benefit of increasing the amount of input DNA must be weighed against our findings that this appears to have a direct toxic effect to the cell lines. If it is found that the amount of DNA is toxic to the cell line, it may be necessary to decrease the amount of input DNA or increase the number of plated cells. In some experiments, we have introduced up to 15 μg of DNA to a 60-mm plate during transfection.

Incubator CO₂ concentrations outside the range of 4.5–5.5% may adversely affect transfection efficiency.

11. In order to increase the relative retroviral titer, decrease the volume in this step to 3.0 mL/60-mm dish. Depending on the gene expressed from the introduced construct, the producer cells may be readily dislodged from the culture dish. It is therefore important to add the media to the side of the dish, taking care not to disturb the adherent cells.

12. The packaging cells should be nearly confluent by 24 h posttransfection. Under these circumstances, the retroviral supernatants should be collected at 48 h posttransfection. If the cells are not confluent by this point, it may be necessary to wait until 72 h. With the transient transfection methods, we find that retroviral titers drop if supernatant is harvested after 72 h posttransfection. If the cells are not confluent by 72 h, decrease the amount of input DNA and/or increase the number of initially plated cells with the goal of obtaining a confluent plate by 48 h posttransfection. In some experiments, we have used conditions in which the plate is almost confluent at the time of transfection and have obtained good results.

It is necessary to wait at least 36 h posttransfection to obtain high-titer retroviral supernatants. At this point, it may be possible to harvest the supernatant every 12–72 h without a significant loss in titer.

If a retroviral vector containing an easily assayable marker, such as βgal, is used, it is possible to stain or FACS the cells 48 h posttransfection to test the success of the transfection. When initially optimizing the system, transfection efficiencies should approach at least 50%.

13. Freezing does not appear to cause more than a twofold drop in titer, as long as the cells do not undergo more than one freeze/thaw cycle. If the viral supernates undergo more than one freeze/thaw cycle, there is a significant drop in retroviral titer.

14. No difference in infection efficiency was found between 4 μg/mL polybrene and 8 μg/mL polybrene. If 8 μg/mL polybrene is used, however, it is suggested that the polybrene be left on the cells for 3–5 h since the higher polybrene concentration is toxic to the NIH3T3 cells. At 4 μg/mL polybrene, no toxicity to the fibroblasts is observed; however, a 5-mL infection cocktail is recommended for overnight infections to prevent dehydration. Primary T-cells are sensitive to high concentrations of polybrene; a range of polybrene concentrations should be checked to find a concentration(s) that provides maximal infection efficiency.

15. When working with nonadherent cells, one has the choice of infecting by adding the retroviral supernatant directly to the cells or cocultivating the nonadherent cells with the retroviral producer cells. The advantage of the latter is that there is
ongoing retroviral production; however, this must be weighed against the disadvantage of harvesting producer cells together with the target cells. Although we have not tried, it may be possible to minimize this problem by irradiating or mitomycin C-treating the producer cells prior to cocultivation. In general, we have obtained higher infection frequencies by cocultivation.

16. For many nonadherent cells, achieving an optimal infection requires growth in the appropriate medium. Because 293 cells and their derivatives appear to tolerate many different medium bases and serum types, it is possible to alter the medium at 24 h posttransfection (see Section 3.4, step 5) so that the resulting retroviral supernatant will be harvested in the appropriate growth medium. When infecting with supernatants derived from 293 cells or derivatives, it should be remembered that these cells may provide a different cytokine/growth factor milieu than the NIH3T3-derived producer cells. A careful analysis of factor production by these cells has not been performed.

We use polybrene at a concentration of 2 μg/mL for infection of nonadherent cells, although it may be possible to increase this concentration to 4 μg/mL without significant toxicity.

17. It is possible to increase infectious titers by approximately twofold by centrifuging and resuspending the cells in a freshly prepared infection cocktail 24 h after infection. We have not tried repeating this procedure more than once.

18. At this point, it is important that disturbance of the packaging cells is minimized. Use extreme care when removing the nonadherent cells from the packaging cells and do not wash the plate at this step. Also, it is unimportant to remove all of the nonadherent cells at this step. The purpose of this step is to add fresh medium and retroviral supernatant without losing nonadherent cells. Sufficient residual media remains on the plate to maintain the cells during the short centrifugation step described in Section 3.8., step 3. With longer centrifugation times, return the culture plates to the incubator.

19. The plate may be washed at this step; however, extreme care should be used so that the adherent cells do not detach. At this step, one is trying to achieve maximal removal of nonadherent cells. With this procedure, contamination by packaging cells is often less than 10%.

20. The method, described by Burns et al. (21), employs initial selection of a stable producer clone. The transient method described here has possible advantages for retroviral constructs that do not express easily assayable genes, or express genes that might be selected against in prolonged culture conditions while isolating a high-titer clone. The method described below uses a βgal expressing retrovirus, but it is expected that once standardized, the user can employ retroviral vectors with nonselectable markers for infection of human and nonhuman target cells. It is possible to substitute other envelope proteins for the VSV-G polypeptide used here.

21. Conditioned media is defined here as media obtained from a subconfluent culture plate of Anjou cells. Plating in conditioned media results in a more rapid attachment of the cells and a uniform cell distribution at 8 h postplating of cells.
22. Within 36 h after transfection, syncytia can be noted forming in the transfected AnJou cell population. β-gal staining of lacZ transfected AnJou cells after removal of viral supernatant accentuates visualization of syncytia formation.

23. Coculturing of the target cells with VSV-G transfection producer cells is not recommended owing to syncytia induction. We have also observed syncytia formation following infection of NIH3T3 target cells using concentrated or unconcentrated (base) virus supernatant. This is likely owing to the carryover of membrane fragments bearing VSV-G, which is known to be highly fusogenic. Continued growth of these cells resulted in a population of singly, normally dividing cells. Initial tests detected no helper virus formation (Caldwell et al., unpublished).

Base titers achieved in this system are up to $5 \times 10^5$ pseudotyped virions/mL. It has been possible to directly scale the transfections into larger 150-mm plates for transient production of larger volumes of virus (50 mL/150-mm plate) and for subsequent concentration. Our initial attempts at concentration resulted in an infectious titer of $2 \times 10^6$ pseudotyped virions/mL. It should be possible to attain higher titers by additional centrifugation.

Acknowledgments

The authors are extremely grateful to David Baltimore for continued encouragement, advice, and support, and in whose laboratory, many of the experiments were conceived and carried out. The authors would also like to thank their colleagues in the Baltimore lab, Nolan lab, and elsewhere for advice and suggestions regarding these protocols. W. S. P. was supported by a Howard Hughes Medical Institute Physician Postdoctoral Fellowship and is a Special Fellow of the Leukemia Society of America. G. P. N. is a Scholar of the Leukemia Society of America and a recipient of the Burrough’s Wellcome New Investigator Award in Pharmacology and is supported by NIH R01AI35304, NIH and a gift from Tularik, Inc.

References


Methods for the Construction of Retroviral Vectors and the Generation of High-Titer Producers

Isabelle Rivière and Michel Sadelain

1. Introduction

1.1. Recombinant Retroviruses as Vectors for Gene Transfer

Retroviral vectors are powerful tools for gene transfer that are useful in the context of experimental as well as clinical applications. Defective recombinant retroviruses allow for efficient gene transfer into a broad range of mammalian cells derived from different species and different tissues. Safe gene transfer can be achieved using helper-free viral stocks, owing to the development of vectors and packaging cell lines that minimize the probability of hazardous recombination events. Retrovirus-mediated gene transfer leads to stable proviral integration in the target-cell genome, a key feature when the foreign genetic material must be faithfully transmitted to the progeny of the transduced parental cell. Murine leukemia virus (MLV)-based vectors accommodate numerous modifications, thus providing a plastic tool that can be tailored for very diverse applications.

There are, nonetheless, limitations to this vector system. Using conventional MLV-based systems, efficient gene transfer is limited to dividing cells expressing the viral receptors. Random proviral integration in the genome, biased toward sites of active chromatin, is prone to causing insertional mutagenesis. The particle packaging constraints limit the size of the inserted sequence to 7–8 kb. Transmission of the vector sequence is subjected to the multiple steps of the retroviral life cycle. In the course of reverse transcription and integration, sequence rearrangements or recombinations that depend on the nature of the juxtaposed retroviral and inserted sequences may occur. Therefore, faithful transmission of the original structure cannot be taken for granted, and every
new construction must be thoroughly characterized. The construction of one particular vector and the generation of a stable high-titer producer is a rather cumbersome procedure that sometimes demands a significant commitment. Nonetheless, a high-titer producer is a convenient and durable reagent.

1.2. Principles of Retroviral Vector Design

Gene transfer systems based on defective viral vectors typically comprise two components: a replication-defective virus bearing the sequences to be transferred and a helper virus providing in trans the replication and packaging functions that allow for propagation of the recombinant genome. In the case of retrovirus-mediated gene transfer, helper-free viral stocks can be generated owing to the availability of recombinant helper genomes that are very inefficiently packaged (1,2). The packaging cell system is described elsewhere in this series (3).

The principles of retroviral vector design are derived from the retroviral life cycle (see refs. 4 and 5 for review). Murine leukemia viruses, which provide the constitutive elements of most retroviral vectors and packaging cells, bind to their target cell via a receptor that determines the host range of the infectious particle. Ecotropic particles bind to a receptor present on mouse and rat cells (6,7). Amphotropic and GaLV-coated particles bind to receptors present on numerous mammalian cells, including primate cells (8). The choice of receptor is determined by the sequence of the env-encoded glycoprotein. After fusion of the viral and cellular membranes, the core particle enters the cytoplasm where the single-stranded RNA retroviral genome is reverse-transcribed into a double-stranded DNA copy bearing direct, long terminal repeats (LTR) at each end. After transport to the nucleus, the viral DNA integrates into the host cell genome of dividing cells (9,10). This proviral DNA is transcribed by host RNA polymerase and yields two RNA transcripts: one full-length transcript, which bears the packaging signal (1), may serve as either the genomic RNA incorporated into nascent virions or as the template for translation of the gag-pol-encoded polypeptides; the second transcript, or subgenomic species, is generated by the splicing of a 5.2-kb intron and serves as the mRNA for the env-encoded products. The gag-pol-encoded polypeptides and genomic RNA transcript assemble in the cytoplasm to form virions that acquire the retroviral envelope glycoprotein upon budding from the infected cell.

Consequently, the guiding principles of vector design are to retain within the vector the minimal cis-acting sequences necessary for genomic transcription, packaging, reverse transcription, and integration, and to avoid recombination between the vector and other viral sequences that could generate a replication-competent genome. The latter stipulation critically depends on
Construction of Retroviral Vectors

the packaging cell design. Recombination events are minimized when the \textit{gag-}, \textit{pol-}, and \textit{env}-encoded polypeptides, coexpressed in the packaging cell, are transcribed from at least two separate plasmids lacking the packaging signal and displaying as little homology as possible with vector sequences \((11-13)\).

The vector itself does not encode any viral product and typically includes the following elements: The LTRs contain essential sequences for reverse transcription and integration in the target cell. The 5' LTR controls vector transcription. Adjacent to the 5' LTR lie three critical elements: the primer-binding site \((I4)\), the packaging signal, \(\psi\) \((I)\), and additional \textit{gag} sequences (altered within the vector so as to prevent translation of any \textit{gag}-encoded peptide). The latter element enhances the packaging of the vector transcript \((15,16)\), defining an extended packaging region, \(\psi^+\). This fragment usually includes a splice donor site, located 5' to the packaging signal in the Moloney-MLV sequence. The 3' end of the vector includes sequences past the \textit{env} stop codon and the 3' LTR that are necessary for reverse transcription and proviral integration.

The U3 region within the 3' LTR of the vector is reverse-transcribed and incorporated in both LTRs generated in the target cell. This property is exploited in different ways in the design of retroviral vectors. For example, deletions in the 3' U3 region will result in transcriptionally weakened LTRs in the target cell. This deletion is useful for the generation of retroviral enhancer traps or the construction of internal promoter vectors, as shown in Fig. 1, top. New sequences, e.g., an entire transcription unit, can be inserted in the 3' U3 region, which therefore will be duplicated in the target cell, albeit with variable reliability. The 3' U3 region of Moloney-MLV can be replaced with the homologous sequence taken from other viruses to generate novel recombinant LTRs in the target cell, as shown for the LTR-driven vector in Fig. 1, bottom.

1.3. Specific Features of Vector Design

1.3.1. LTR-Driven Expression

The U3 region of the murine leukemia viruses comprises an enhancer and a promoter that together act as a strong transcriptional element. The LTR-driven vectors provide relatively high expression of the transferred gene in a broad range of differentiated or transformed mammalian cells \((18-20)\). Expression levels seem enhanced in vectors that incorporate functional splicing signals including either cryptic \((15)\) or engineered \((17,18)\) splice acceptor regions. The vectors N2 \((15)\) and MFG \((18,20,32,33)\) shown in Fig. 2 are representative of this category. However, LTR-driven vectors do not func-
Fig. 1. Schematic representation of the structure of Moloney-MLV (left), internal promoter vectors (top), and LTR-driven vectors (bottom). D, splice donor; A, splice acceptor; ψ, packaging signal; AAA, polyadenylation tract, ☐☐☐ internal promoter, ☐ gene of interest.

7.3.2. Constitutive Internal Promoters

Promoters other than the LTR can be incorporated into retroviral vectors. Viral promoters, such as SV40, CMV, HSV-TK (15,26,27), and mammalian promoters, such as phosphoglucom kinase (PGK), histone 4, or β-actin (28–30), are often used. Transcription from the LTR is minimized by crippling the U3 region of the 3' LTR, most commonly by deletion of the enhancer sequence, as described in Section 1.2. These vectors confer constitutive and nontissue-specific expression, like LTR-driven vectors, but differ with respect to their mean level of expression in different tissues, their ability to be expressed...
Fig. 2. Examples of retroviral vector design. N2 (15), MFG (18), HSGneo (29), Mβ6L (36), LXSN (27), and d-cistronic MFG (41). The vector sequences are derived from Moloney-MLV unless otherwise indicated: SD, 5' splice donor; 3' SA*, sequence from NdeI to NlaIII containing the 3' splice acceptor (SA); SA**, cryptic SA; Mo-MuLV, sequences derived from Moloney murine leukemia virus; Mo-MuSV, sequences derived from the Moloney murine sarcoma virus; LCR, locus control region; Enh−, deletion in the enhancer sequences; SV40 E/P, SV40 promoter and enhancer sequences, ψ, packaging signal; ψ+, extended packaging signal; IRES, internal ribosome entry site; H4 Pr, histone H4 promoter; β Pr, β-globin promoter, P, PstI; Nr, NarI; Xh, XhoI, N, NdeI; B, BglII, X, XbaI, Nl, NlaIII; H, Hpal, C, ClaI.
in embryonic cell types, their capacity to sustain long-term expression, and their susceptibility to position effects.

1.3.3. Tissue-Specific Expression

Tissue-specific expression is usually achieved by targeting to a specific cell type a nontissue-specific expression vector, as in the case of hepatocytes (31), myoblasts (32), or synoviocytes (33). When it is not possible to directly target a particular cell type, or when infection has to be directed to a multipotential precursor cell, or if promiscuous promoters fail to sustain expression in the relevant cell population, tissue-specific transcriptional elements must be considered. Several internal promoter/enhancer combinations have been inserted in retroviral vectors, e.g., muscle- or erythroid-specific vectors (34,35). The case of β-globin expression is of particular interest and is probably the most extensively studied tissue-specific vector. In order to treat β-globin disorders by gene addition, it is necessary to incorporate a recombinant β-globin transcription unit that confers high-level, erythroid-specific, stage-specific, and position-independent gene expression, into a vector suitable for efficient transfer into pluripotential hematopoietic stem cells. Moreover, as the β-globin cDNA fails to direct sufficient expression, the β-globin gene, with its two introns, must be incorporated in the vector. Inclusion of the β-globin gene with its promoter, and two proximal enhancers leads to erythroid-specific expression. However, the resulting expression levels are very poor (35), thus establishing that tissue-specific or regulated expression is unlikely to be achieved by merely incorporating a tissue-specific promoter. Over 150 different constructions, varying in their β-globin gene length, transcription regulatory elements, and viral sequences had to be tested before a vector yielding high-titer viral stocks and conferring enhanced erythroid-specific expression was obtained (see Section 1.4.2. and ref. 36).

1.3.4. Dual Promoter Vectors

There are several ways to achieve dual gene expression from one vector. The most common is to engineer dual promoter vectors (15,17,27). Typically in such a vector the expression of one gene is under LTR transcriptional control and the other, inserted 3’ to the stop codon of the first, is under the control of an internal promoter/enhancer combination. One example is LXSN (27) in which the gene of interest is driven by the LTR, whereas the gene encoding neomycin phosphotransferase (neoR) is driven by SV40 transcriptional elements (see Fig. 2). Despite the potential for transcriptional interference (37,38), this type of vector, which bears two strong and nontissue-specific promoters, is very widely utilized.
1.3.5. Dicistronic Vectors

The internal ribosomal entry site (IRES) isolated from (+) strand RNA picornaviridae (39) can be inserted between two coding sequences to ensure gene coexpression in different cell types (e.g., ref. 40). Thus, dual gene expression may be achieved using single transcription unit vectors, as shown in Fig. 2. We have found that such vectors are transmitted faithfully, yield high titers, and ensure gene coexpression in different cell types (41). Triple gene transfer may be achieved by building tricistronic vectors (42) or dicistronic vectors, including a fusion gene that encodes two functions. Because IRES function depends on cellular factors and may be affected by flanking sequences, it is necessary to establish for every new vector that gene coexpression is faithfully achieved in the tissue of interest.

1.3.6. Future Directions

Today, there are many retroviral vectors available, as illustrated above, yet there is still room for much improvement. Achieving regulated expression in vivo, especially with respect to tissue specificity and inducibility (55) of gene expression, and persistent gene expression over the long term in vivo (18), are major objectives. The generation of vectors that express at any proviral integration site, i.e., that overcome position effects, may be achieved by inclusion of chromatin regulators such as locus control regions and insulators (36). Other major ongoing efforts aim to construct new packaging cell lines that yield higher titer virus stocks, or generate particles optimized for human cells (56) or specifically targeted to one given tissue. The development of retroviral vector systems that allow for the infection of nondividing cells will vastly extend the use of retrovirus-mediated gene transfer (54) when packaging cells are available.

1.4. Generation of High-Titer Retroviral Producers

The titers obtained from producers generated by DNA transfection vary widely on a clonal basis. It is therefore useful to screen several clones to identify the highest titer packaging cell line. Moreover, a clone is a more stable reagent than a pool of producers that is gradually taken over by the fastest growing cells that are not necessarily those with the best titer. Several methods are available for comparing clones for their particle output. These methods range from the determination of viral RNA content in the producer cell supernatant, to proviral copy number determination in generic target cells, to expression studies in generic or specific target cell populations (see Section 3.2.). Before undertaking this screening, certain strategies should be considered to increase the likelihood of generating a high-titer producer.
1.4.1. Cross-Infection and Ping-Pong Strategies

As the titer can be limited by the production of the vector transcript within the packaging cell, it may be useful to introduce multiple copies of the template in sites of active chromatin. The introduction of the construct by infection rather than by transfection will favor integration into sites of active chromatin. It will also eliminate the transcriptional interference that may occur in concatameric inserts. This infection can be achieved by repeated infection of the producer cell, if the cells are permissive to superinfection. Viral interference caused by expression of the env-encoded glycoprotein can be circumvented by superinfecting an amphotropic producer with recombinant ecotropic viral particles and vice versa (27,43). Cross-infections can be repeated several times, going back and forth between amphotropic and ecotropic packaging cells (ping-pong). However, all crossinfections increase the risk of generating replication competent genomes (12,44). Cocultivation of ecotropic and amphotropic packaging cells is definitely not recommended (45).

1.4.2. Identification of Sequences that Reduce Titers

Some inserts result in vectors consistently yielding low-titer viral stocks, whatever retroviral vector backbone is used. Such is the case for vectors bearing the human β-globin gene (16,35). In contrast, incorporating the human β-globin cDNA sequence in the same vectors yields high titer viral stocks. A panel of vectors bearing recombinant human β-globin genes lacking untranslated sequences was generated (gene lacking intron 1, gene lacking intron 2, and so on) and transfected in the ψ-CRE packaging cell line (36). Transmission of the vector structure to NIH 3T3 fibroblasts was studied by semiquantitative Southern blot analysis as described in Section 3.2.2. The result of this study is shown in Fig. 3. Very low titers are achieved with constructs bearing intron 2 (lanes A–D). The titer is comparable to the titer of the cDNA vector when intron 2 is entirely deleted (lanes E–G) or replaced by the human α1-globin intron 2 (lane J), but only partially increased by subintronic deletions (lane I). This analysis is achieved using pooled producers, analyzed side by side. It therefore reflects the mean transmission achieved by each vector. This systematic study has led to the identification of sequences within intron 2 that dramatically reduce titers, thus leading to the rational design of high titer and genomically stable β-globin vectors that bear a modified β-globin gene (36). The comparison of several clones by this assay, which relies on Southern blot analysis, is also useful to screen for the highest titer producer (see Section 3.2.).

1.4.3. Toxic Gene Expression Precluding the Generation of Stable Producers

Expression of certain genes is expected to interfere with the function or proliferation of the packaging cell, e.g., interferons or antiproliferative genes.
Fig. 3. Example of vector copy number determination by Southern blot analysis: comparison of vector transmission between β-globin vectors bearing different deletions in the human β-globin gene (see text). Genomic DNA (packaging cell, left lane, target cell, right lane) was digested with NheI (LTR to LTR digest) and equal amounts were loaded onto a gel. After transfer, the blots were probed with a human β-globin probe (36).

In this case, the generation of stable producers will require the design of regulated and/or tissue-specific expression vectors that repress gene expression in fibroblasts. Otherwise, it may be necessary to utilize transient producers (see Chapter 3).

2. Materials

2.1. Cells

1. Retroviral packaging cell line (1–3, 11–13, 46, 47).
2. NIH 3T3 fibroblasts.
3. 3T3-116 or 3T3-BAG cells: 3T3-116 cells and 3T3-BAG cells harbor in their genome defective recombinant retroviruses expressing the histidine dehydrogenase (his) and the β-galactosidase gene, respectively (3).

2.2. Cell Culture

1. Selective and nonselective tissue-culture media.
2. DMEM, calf serum (Gibco-BRL, Grand Island, NY).
3. Penicillin, streptomycin, G418 (Gibco-BRL).
5. 96-, 24-, and 6-well plates, 10-cm diameter plates.
6 Cloning rings placed in a glass Petri dish coated with petroleum jelly or vacuum grease, sterilized by autoclaving

2.3. Solutions and Materials

2.3.1. Calcium Phosphate Precipitate

1. HBS: 137 mM NaCl, 5 mM KCl, 0.7 mM Na$_2$HPO$_4$, 6 mM dextrose, 21 mM HEPES Na$^+$ (pH 7.05; adjust with 1N NaOH). Filter-sterilize and store at 4°C.
2. 2M CaCl$_2$. Filter-sterilize and store at 4°C
3. 15% Glycerol in HBS.

2.3.2. Electroporation

1. 0.4-cm Bio-Rad (Hercules, CA) electroporation cuvet 165-2088.
2. Electroporation power device, Bio-Rad Gene Pulser

2.3.3. Infection

1. Polybrene, stock solution 800 μg/mL (Sigma, St. Louis, MO; cat. no. H9268) in PBS$^-$. Filter-sterilize and store at 4°C.
2. 10-mL Disposable plastic syringes.
3. 0.45-μm Disposable filters (not 0.22-μm)

3. Methods

3.1. Transfection and Selection of Virus-Producing Clones

Producers of recombinant retroviruses are obtained by transfecting any of a variety of retroviral packaging cell lines (1–3,11–13,46,47). There are a number of transfection protocols yielding stable transformants, such as calcium phosphate coprecipitation, electroporation, or lipofection. For constructs lacking a selectable marker, cotransfection with a plasmid such as pSV2neo (48) is necessary. Other selectable markers can be used (reviewed in ref. 49), depending on the markers present in the packaging cell line used (3). A 10-fold molar excess of the retroviral plasmid should be used to ensure that stable selected transfectants have taken up both plasmids. Transfected packaging cells should be transferred into appropriate selective media 24 or 48 h after transfection to allow for integration and expression of the selectable marker gene. After the selection period, individual colonies are isolated. If cloning rings are used, colonies should be sparse enough on the plate to avoid cross-contamination between adjacent colonies. The subcloning can also be performed by limiting dilution in 96-well plates, seeding 0.5–2 cells/well. About 30 colonies are picked and expanded in 24- and 6-well plates, and finally 10-cm plates. At that stage, the viral titer can be assayed and at least one vial per clone should be frozen and stored in liquid nitrogen.
The methods described herein are used to generate high titer producers from the \( \psi \)-CRE and \( \psi \)-CRIP packaging cells (12).

### 3.1.1. Calcium Phosphate Transfection: Method A

1. **Day 1.** Seed the packaging cells \((5 \times 10^5 \text{ cells})\) in 10-cm diameter tissue-culture plates in DMEM, 10% calf serum, penicillin-streptomycin medium.
2. **Day 2:** Mix 10–15 \( \mu \)g of vector DNA and 1 \( \mu \)g of the plasmid DNA carrying the selectable marker (10:1 molar ratio), both at concentrations of 0.5–3 \( \mu \)g/mL, in 0.5 mL HBS. Add 31 \( \mu \)L of 2\( M \) CaCl\(_2\) and flick with finger to mix.
3. Incubate at room temperature for 45 min. A faint precipitate should appear.
4. Remove the medium from the packaging cell line. Add the precipitate and incubate the plates at room temperature for 30-40 min. Rock the plates at 10-min intervals.
5. Add back 10 mL of medium and incubate at 37°C for 4 h.
6. Remove medium and wash once with fresh medium.
7. Optional (see Note 1): Add 2.5 mL of 15% glycerol in HBS and incubate at 37°C for 3 min. Remove glycerol and rinse cells once with 10 mL of medium.
8. Add back 10 mL of medium.
9. Incubate at 37°C for 24-48 h.
10. **Day 3 or 4:** Split cells at 1 to 5, 1 to 10, 1 to 20, 1 to 40 into the appropriate selective media. Re-feed cell cultures with drug-containing media every 3 d. Examine the cultures under a microscope to determine the efficiency of drug selection.
11. Choose the best plates for isolating individual clones, either by picking individual colonies using cloning rings, or by limiting dilution (seeding 0.5–2 cells per well in 96-well plates).

### 3.1.2. Electroporation: Method B

We provide here the parameters for the packaging cell lines \( \psi \)-CRE and \( \psi \)-CRIP (also suitable for 3T3 fibroblasts). Although most of the packaging cell lines are derived from 3T3 fibroblasts, it is recommended to optimize parameters for the specific packaging cell line used.

1. Trypsinize the packaging cell line and resuspend the cells at \( 2 \times 10^7 \text{ cells/mL} \) in DMEM, 1% calf serum.
2. Place 250 \( \mu \)L of the cell suspension in 0.4-cm electroporation Bio-Rad cuvet on ice for 10 min.
3. Add to the cell suspension 20–50 \( \mu \)g of mixed vector DNA and plasmid carrying the selectable marker (both linearized; see Note 2) at a molar ratio of 5:1. Incubate on ice for 10 min.
4. Set up the electroporation apparatus according to the manufacturer’s instructions and subject cell/DNA mixture to the electric field (960 \( \mu \)F and 200 V).
5. Return cell/DNA mixture on ice and incubate for 5 min.
6. Plate cells in nonselective medium in 10-cm plates. Proceed to steps 9–11 of Section 3.1.1.
3.1.3. Lipofection: Method C

It is also possible to obtain stable transformants by lipofection. This method is described in details elsewhere (50).

3.1.4. Transient Transfection: Method D

This method is described in detail in Chapter 3.

3.2. Isolation of the Best Producer Clones

The viral titer reflects the rate of release of infectious retroviral particles from the producer cells, but there is no direct method to assess it. Consequently, viral titers are generally derived by estimating the concentration of infectious particles in the producer cell supernatant harvested under defined conditions. This supernatant is typically tested on cells highly susceptible to retroviral infection such as NIH 3T3 fibroblasts. Other indicator cells can be used but the read-out of the viral titer is affected by the susceptibility of the target cells to viral infection (see Note 3). There are several ways of estimating titers or assaying for vector transmission. We will emphasize here the quantification of proviral copy number per target cell by Southern blot analysis. After identification of the highest titer clones, two additional tests must be carried out: verification of expression of the transgene and screening for production of helper virus by the clonal producer.

3.2.1. RNA Dot Blot Analysis

The detection of retroviral RNA in the supernatant of the virus-producing cell lines by RNA dot blot (51) is not quantitative, nor does it provide information on the structure of the encapsidated vector. However, this method can be used for an initial screening, prior to further characterization of the clones that generate the strongest signals.

3.2.2. Titration on NIH 3T3 Cells Fibroblasts

1. Day 1. Seed the virus-producing cells so that they will almost reach confluence 48 h later. This is done by placing about $5 \times 10^5$ cells in a 10-cm plate (the exact seeding density depends on the growth rate of the packaging cell clone and needs to be determined).
2. Day 2: Replace the medium on the producers with 10 mL of fresh medium.
3. Plate NIH 3T3 cells at $5 \times 10^5$ cells in 10-cm dishes.
4. Day 3. Harvest the supernatant from almost confluent monolayers of producer cells and filter through 0.45-μm filters (see Note 4).
5. Infect NIH3T3 fibroblasts with 5 mL of undiluted and diluted viral supernatant added of 8 μg/mL of polybrene (see Note 5). Incubate the plates at 37°C for 4 h. Then replace the supernatant with 10 mL of fresh medium and incubate at 37°C for another 48–72 h.
6. Day 5. Depending on the method chosen to evaluate the virus titer, one can start selection if the retroviral vector carries a selection marker (see Section 3.2.2.3.) or prepare the infected NIH 3T3 cells for either FACS analysis or protein expression analysis (see Section 3.2.2.2.), or extract high-mol-wt DNA to perform Southern hybridization analysis of integrated proviral genomes (see Section 3.2.2.1.)

7 Select 2 or 3 high-titer viral producer cell clones (see Note 6). Freeze at least 10 vials of the selected clones and check these for helper-virus (see Section 3.3.)

3.2.2.1. Titration of Producer Cell Lines Based on Proviral DNA Analysis: Method A

Proceed to the extraction of genomic DNA from the target cells (52) at 48–72 h after infection. Restriction enzyme digestion and Southern blot analysis are performed using standard procedures (53). The restriction enzyme used should cut in both LTRs of the proviral genome. The probe should not overlap with the retroviral vector sequences.

Load gels with 10 μg of digested genomic DNA per lane. As control for copy number, one can use either plasmid DNA or, ideally, genomic DNA from a cell line carrying one copy per cell of the same retroviral plasmid. All DNA samples are digested with the same restriction enzyme. To determine the number of proviral genome per infected cell using plasmid DNA as control, mix 10 μg of DNA from noninfected NIH 3T3 cells with amounts of plasmid corresponding to 5, 1, 0.5, and 0.2 copies per cell. The amount of plasmid corresponding to 1 copy per cell in 10 μg of genomic DNA is given by the following formula: [plasmid size] x 10/[genome size*]. (*) = 6 x 10^9 bp for the mouse genome.

In this assay, we consider a clone to be a high titer producer when 0.5 or more integrated copies per cell are detected, using a fivefold dilution of the supernatant to infect NIH 3T3 fibroblasts. We have successfully used such high-titer clones for the efficient infection of murine bone marrow cells as shown in vivo in long-term bone marrow chimeras (18), infection of primary lymphocytes (see Chapter 17), embryonic stem cells, and primary murine macrophages (I. Rivière, unpublished observations).

3.2.2.2. Identification of the Highest Titer Producer by Expression Studies in Target Cells: Method B

In some instances, the titer of different clones can be compared by measuring the fraction of target cells expressing the transferred gene. This can be easily done by FACS analysis when the gene encodes a cell surface protein against which specific antibodies are available (see Chapter 17). When the protein is detectable using a bioassay, one can compare levels of expression of the transferred gene in target cells infected with different viral stocks. Conse-
sequently, the investigator will choose to either directly screen all the clones with the procedure from Section 3.2.2.2., or first proceed to Section 3.2.2.1. and subsequently to Section 3.2.2.2. for a few clones.

3.2.2 3. Titration Based on the Expression of a Selectable Marker. Method C

This method is the most commonly used method, providing that the vector contains a selectable marker. The read out, i.e., the enumeration of drug-resistant colonies, is not a simple measurement of particle formation and gene transfer because it also depends on the expression characteristics of the vector. This method is described in this series (54).

3.3. Detection of Helper Virus

The presence of replication-competent virus generated by recombination events occurring in the producer cell line or in the target tissues must be systematically monitored. There are several ways to assay for helper virus. The mobilization assay consists in challenging a cell line harboring a defective recombinant provirus with the viral stock or the fluid (plasma for example). If helper virus is present, the transcribed defective recombinant provirus will be packaged into retroviral particles (mobilization process) that will appear in the culture media of the challenged cells. The mobilized provirus carries a selectable marker (histidinol dehydrogenase in 3T3-116) or a reporter gene (β-galactosidase in 3T3-BAG) that can be detected by infecting target cells such as NIH 3T3 fibroblasts.

3.3.1. Mobilization Assay (adapted from ref. 3)

1. Day 1–3: Prepare the supernatant of the virus-producing cell lines as described in Section 3.2.2., steps 1, 2, and 4
2. Day 2: Seed the indicator cell line (3T3-116 or 3T3-BAG, see Section 2.) at $5 \times 10^5$ cells in 10-cm plates.
3. Day 3: Infect the indicator cell line with 5 mL of each viral stock added with 8 μg/mL polybrene. Incubate the plates at 37°C for 4 h. Replace the infection medium with 10 mL of fresh medium and incubate at 37°C for 48 h
4. Day 5–7: Split the infected indicator cells and prepare supernatant from these cells as in Section 3.2.2., steps 1, 2, and 4.
5. Day 6: Plate NIH 3T3 cells at $5 \times 10^5$ cells/10-cm plate.
6. Day 7: Replace the medium on the NIH 3T3 cells with 5 mL of undiluted and diluted supernatant from the indicator cell line (10-, 100-, and 1000-fold dilutions). Add polybrene to a final concentration of 8 μg/mL and incubate for 4 h. Next, replace the medium with 10 mL of fresh medium and incubate for 48 h at 37°C.
7. Day 9: Split the challenged NIH 3T3 cells at 1 to 10 in selective media, or stain for β-galactosidase activity, according to the indicator cell line used (see Section 2.)
3.4. Culturing the Selected Virus-Producer Cell Lines

At least 10 vials of the selected virus-producer cell clones should be frozen in culture medium containing 10% DMSO. If the cells need to be grown for a long period of time, thaw a new vial from the original stock every month.

3.5. Boosting the Titer and Improving the Infection Efficiency

A certain number of approaches can be undertaken if the highest titer producer cell line selected does not allow for infection of the target cells at a sufficient efficiency.

3.5.1. Virus Concentration

The concentration of the virus by centrifugation is sometimes used, but this procedure adversely affects viral infectivity. This method is described in this series (53). VSV-G coated particles are more resistant to virus concentration procedures (58).

3.5.2. Cross-Infection and Ping-Pong

The primary producer cell lines obtained by methods described in Section 3.1. can be used to infect another packaging cell line. This can be achieved by a single cross-infection, which is less likely to generate helper virus than ping-pong (see Section 1.4.1.), yet increases this risk relative to that associated with transfection. As ecotropic virus producers are refractory to infection by ecotropic virus but not amphotropic virus, and vice versa, the tropism of the primary producer cell line must be different from that of the packaging cell line to be used.

Use the protocol described in Section 3.2.2. to perform the infection of the secondary packaging cell line (e.g., the ecotropic supernatant is used to infect an amphotropic or GaLV packaging cell line). The highest titer producer is selected as for the primary producers.

The titer can be increased by mixing ecotropic and amphotropic packaging cell lines at a ratio of 1:1. Recombinant retroviruses repeatedly infect the two retroviral packaging cell lines, leading to increased proviral copy integrations, increased virus titer, and generation of replication-competent helper virus (45). We do not recommend this method.

3.5.3. Cocultivation of the Target Cells with the Producer Cell Line

Cocultivation of nonadherent target cells on a monolayer of producers usually results in greater gene transfer efficiency as compared to infection using viral stocks, provided that suitable culture conditions be defined. This method is described in Chapters 16 and 17, dealing with the infection of nonadherent cells (see also Note 6).
4. Notes

1. Glycerol shock: This step is optional but improves the transfection efficiency in a twofold range.
2. Linearization of plasmids for electroporation. Linearized DNA is more recombinogenic than supercoiled or nicked circular DNA and increases the number of stable transformants.
3. The susceptibility to infection of the target cells depends itself on various parameters such as viral receptor expression on the cell surface, expression of endogenous retroviral envelope-like products, and the rate of cell division.
4. The titer is determined on a 16–24 h conditioned medium. Do not use 0.22-μm filters to prepare the viral stock. Filtered supernatant can be frozen at −70°C for several months with gradual loss of retroviral particle infectivity.
5. It is suggested to infect the cells with two concentrations of retroviral particles, using for example the harvested viral stock undiluted and diluted 1:5 in the same culture medium.
6. If the target cells are infected by cocultivation (for example with hematopoietic cells), some virus-producing cell clones might be more suitable than others for the survival/cycling status/infection of the target cells. This is related to the intrinsic characteristics of each clone, such as cytokine production, rate of division, and metabolism of the cells, which lead to variability in the content and the pH of the supernatant. Thus, two clones with the same titer as determined on NIH 3T3 fibroblasts may differ with respect to gene transfer efficiency into another target cell type.

Acknowledgments

We thank David A. Sanders for reviewing this manuscript, Richard C. Mulligan for his support, and Vida Petromis for help in the preparation of the manuscript.

References


Development of Replication-Defective Herpes Simplex Virus Vectors

David Krisky, Peggy Marconi, William F. Goins, and Joseph C. Glorioso

1. Introduction

Numerous diseases of the nervous system result from single gene or multifactorial gene defects such as cancer, immune pathological disorders, metabolic diseases, and common neurodegenerative syndromes (Parkinson’s and Alzheimer’s diseases). A greater understanding of the molecular, biochemical, and genetic factors involved in the progression of a specific disease state has led to the development of genetic therapies using direct gene transfer to ameliorate the disease condition or correct a genetic defect in situ. Standard gene therapeutic approaches employing retroviruses have not proven feasible for treating disorders of the central nervous system (CNS) since these vectors require dividing cells for integration and expression of the transgene, whereas CNS neurons are postmitotic, terminally differentiated cells. Thus, methods for delivery and expression of therapeutic gene products to treat CNS disease will require new delivery strategies and vehicles including the development of novel vectors for direct gene transfer. These vectors should: efficiently deliver the therapeutic gene(s) to a sufficient number of nondividing neurons; persist long-term in a nonintegrated state within the nerve cell nucleus without disturbing host cell functions; and be able to regulate therapeutic gene expression for diseases that may either require high-level transient transgene expression or continuous low level synthesis of the therapeutic product.

Herpes simplex virus type-1 (HSV-1) possesses a number of practical advantages for in vivo gene therapy to brain and other tissues including its ability to infect a wide variety of both dividing and postmitotic cell types; its ability to be propagated to high titers on complementing cell lines; a large
genome size (152 kb) that enables the virus to accommodate large or numerous therapeutic gene sequences (>35 kb); and the natural biology of HSV-1 infection involves long-term persistence of the viral genome in a latent, nonintegrated state in neuronal cell nuclei (1-3) and other postmitotic cell types in the absence of viral protein synthesis, genome integration, or interference with host cell biology. Since the virus does not disrupt normal host cell biology or express viral antigens during latency, cells harboring latent virus will not be attacked by the host’s immune system and should allow persistence of the viral genome for the lifetime of the host, obviating the need for repeat dosing of the virus vector. During latency, the viral genome is transcriptionally silent except for the expression of a novel set of viral latency-associated transcripts (LATs) (4-9). Since the LATs are not required for the establishment or maintenance of this latent state (10-16), it should be possible to delete these genes and replace them with the desired therapeutic gene to drive expression of this gene product from an otherwise quiescent genome using the novel latency and neuronal cell-specific promoter complex resident within the vector genome.

1.1. The HSV-1 Life Cycle

The HSV-1 particle (Fig. 1A) is composed of: an envelope containing 12 glycoproteins that allow the virus to attach to and infect cells by fusion of the virus envelope with the cell surface membrane (for review, see refs. 17 and 18); a tegument matrix inside the envelope that is composed of viral structural components involved in shut-off of host protein synthesis (19-22), activation of immediate early viral gene expression, and assembly functions (23-28); a regular icosahedral-shaped nucleocapsid (29); and a linear, double-stranded DNA genome containing approx 78 open reading names (30-32). The 152-kb viral genome is segmented (Fig. 1B) with each of its long (U1) and short (U3) unique segments flanked by inverted repeats (IRs). The viral functions have been categorized as to whether they are essential for virus replication in cell culture or are accessory (nonessential) functions that contribute to virus replication and spread in vivo. The viral genes are arranged in a manner such that many of the essential functions or accessory genes tend to be clustered within the genome. Additionally, since very few HSV-1 genes are spliced and the virus possesses a highly evolved recombination system, it is relatively easy to manipulate and engineer the virus for purposes of gene transfer, deleting individual genes or blocks of genes that may play a role in vector toxicity.

During normal infection, HSV-1 infects the skin following direct contact and undergoes a productive (lytic) infection in skin fibroblasts and epithelial cells (Fig. 2A). The lytic replication cycle of the virus takes place in the nucleus (17,33) of the infected cell with the viral genes expressed in a highly regulated cascade (34) of coordinated gene expression consisting of three stages; imme-
Fig. 1. Herpes simplex virus type-1 (HSV-1) particle, genome structure, and gene organization. (A) HSV-1 virion schematic showing the envelope containing virus-specific glycoproteins surrounding the dense tegument layer that encompasses the icosahedral-shaped nucleocapsid possessing a single copy of linear, double-stranded viral DNA. (B) Diagram of the HSV-1 prototype genome depicting the location of essential and accessory (nonessential) viral genes.
Fig. 2. HSV-1 life cycle. (A) Schematic diagram of the HSV-1 lytic replication cycle in vivo. The HSV-1 enters mucosal or epithelial cells and proceeds through the lytic replication pathway. The resulting progeny virus particles can encounter and fuse with the cell surface membrane of peripheral nerve termini that innervate the site of primary infection. The viral nucleocapsid then travels via retrograde axonal transport to the neuronal cell body where the virus can either proceed through the highly regulated cascade of lytic gene expression, or enter latency during which the viral lytic gene program is interrupted and the latency-associated transcripts (LATs) are the sole viral RNAs expressed. In response to a wide variety of stimuli, the virus is capable of reactivating from the latent state, entering the lytic portion of the HSV life cycle at which time progeny virions can either be transported back to the site of the primary infection or the virus may enter the CNS. (B) Temporal cascade of HSV-1 gene expression detailing the roles of HSV-1 IE gene transactivators ICP4, ICP27, ICP0, and the IE promoter stimulatory molecule VP16. The immediate early (IE or α) genes are expressed immediately upon infection in the absence of de novo protein synthesis. The VP16 (αTIF, Vmw65) virus tegument protein interacts with the cellular factor Oct-1 to positively regulate the expression of the IE genes by binding to their promoters. The IE gene products ICP4, ICP27, and ICP0 are responsible for activating early (E or β) genes. Following viral DNA replication, the ICP4 and ICP27 IE polypeptides regulate the expression of the late (L or γ) genes.
diate early (IE or α), early (E or β), and late (L or γ) (Fig. 2B). Three of the
viral IE genes are transcriptional activators that induce expression of E and L
genes (35–42). Early gene functions participate in viral DNA replication, which
must proceed in order for late gene expression to occur (43,44). The late gene
products are largely structural products comprising the nucleocapsid, tegument,
and viral envelope glycoproteins. Viral particles are assembled within the
nucleus, bud from the nuclear membrane, and particle maturation proceeds
during migration through the golgi followed by egress from the cell. Following
focal replication of the virus in these permissive cell types, the virus invades
the nervous system by directly infecting axon terminals of local sensory neu-
rons of the peripheral nervous system (PNS) (45,46). The virus nucleocapsids
are transported in a retrograde manner back to the neuronal cell body where the
virus can either replicate or enter latency. During latency, the linear viral genomes
 circularizes, becomes methylated, and forms a higher order nucleosomal struc-
ture (1–3). At this point, the lytic genes become inactive and the viral latency
transcripts (LATs) are readily detected by in situ hybridization using LAT spe-
cific riboprobes (6–9,15). The virus can remain within the latent state for the
lifetime of the individual, or it can be induced to reactivate from latency by a
variety of stimuli resulting in the resumption of the lytic cycle and the subse-
quent synthesis of progeny virions that may traverse the axon by anterograde
transport establishing an active infection at or near the site of primary infection.

1.2. Engineering HSV-1 Vectors

The optimal HSV vector should be: safe and completely devoid of replica-
tion-competent virus; noncytotoxic; incapable of affecting normal host cell
biology; able to persist in the neuronal cell body in a nonintegrated state; and
capable of expressing the therapeutic gene(s) to appropriate levels at the proper
time(s). The two major considerations in the design of HSV-1 vectors concern
the elimination of the cytotoxic properties of the virus and the development of
promoter systems for proper expression of the therapeutic gene. Considerable
effort has been put forth into addressing both of these issues of vector design.
In this work we will concentrate on the engineering of HSV-1 vectors deleted
for essential gene functions that display reduced toxicity following infection.

1.2.1. Solving the Problem of HSV-1 Cytotoxicity

Since UV-irradiated virus displays substantially reduced cytotoxicity in vitro
(48,49) and disruption of viral IE gene expression by interferon also reduces
toxicity, it is presumed that the cytotoxicity of HSV-based vectors results from
the expression of HSV-1 gene products. The fundamental approach to design-
ing HSV-1 vectors with reduced toxicity is to remove the essential IE genes
of the WLLS, as well as several nonessential genes, whose products interfere with host cell metabolism and are part of the virion (tegument) structure. Deletion of the two essential IE genes that encode the infected cell proteins 4 (ICP4) and 27 (ICP27), blocks early and late gene expression (35,39) (see Fig. 2B). These deletions require that the missing functions are supplied in trans using a complementing cell line (35,50). In order to ensure that recombination does not occur between the defective virus and the viral sequences present within the complementing cell line during propagation, it is essential that these sequences do not share homology with sequences present within the viral genome, and that the deletion of the IE genes from the virus exceed the limits of the complementing sequences. Since many of these viral IE genes are toxic to cells, expression of the complementing genes must be inducible upon infection with the defective virus. This is achieved through the use of HSV-1 IE promoters to drive expression of these toxic transactivating genes from the cell, since these promoters have been shown to respond to the HSV-1 transactivator VP16 (Vmwm or α-TIF) (24–28), a virion tegument component that accompanies the viral DNA molecule into the nucleus of the infected cell. VP16 recognizes a consensus sequence (octomer-TAATGARAT) located at various sites in all HSV-1 IE promoters and together with the cellular transcription factors, octomer binding protein one (Oct-1) and HCF (also termed C1, VCAF-1, and C2F) (51–55), transactivates the IE gene promoters (56–59). In the absence of virus infection, the complementing IE genes in the cell chromosome are silent. However, upon infection with the replication-defective mutant, the VP16/Oct-1/HCF complex transactivates the IE promoters upstream of the complementing viral sequences in the cell line, thereby inducing expression of the necessary products for propagation of the deletion virus.

A third IE gene of interest is ICP0, which is both cytotoxic and capable of promiscuous transactivation of a variety of cellular genes (60–63) as well as enhancing the level of expression of other viral genes (64). ICP0 appears to cooperatively collaborate with ICP4, for example, to increase the activity of this key viral function (65,66), although ICP0 is not a promoter binding protein and thus appears to stimulate an event prior to direct promoter activation (67). Although ICP0 is a nonessential viral function, deletion of ICP0 results in decreased viral titers (40,68), and propagation of high-titer stocks of virus deleted for ICP0 in conjunction with the essential IE genes will require the production of a cell line capable of complementing ICP0, as well as ICP4 and ICP27. Generation of such a line has been extremely difficult, since even low-level synthesis of ICP0 is toxic to the host cell. The two remaining IE genes, ICP22, which affects the phosphorylation of RNA polymerase II (69) and ICP47, which affects the processing of MHC class I antigens (70,71), may also need to be deleted depending on the specific therapeutic application.
In addition to the viral IE gene functions, two additional genes represent targets of interest for removal to reduce toxicity. The infecting virus carries in with the particle a tegument component that has a virion-associated host shut-off (vhs) activity \((19,20)\). The vhs appears to interfere nondiscriminately with mRNA stability \((21)\). Removal of the UL41 (vhs) gene does not affect viral replication \((22)\), but enhances the health of the cell on infection with nonreplicating viral mutants \((72)\). Finally, the virus encodes its own ribonucleotide reductase large (RR1) and small (RR2) subunit gene products. The large subunit (RR1) is expressed both at IE as well as E times during replication owing to its unusual IE-E hybrid promoter \((73)\). Because RR1 also has an associated protein kinase activity \((74)\), it is also prudent to delete this gene.

It is anticipated that deletion of these six genes (ICP4, ICP0, ICP27, ICP22, vhs, and RR1) will essentially shut down viral gene expression upon infection of noncomplementing cells, rendering the virus safe, noncytotoxic, and capable of establishing latency in many if not all nondividing cells. Moreover, the retention of expression of ICP47, recently shown to down-regulate MHC class I antigen expression \((70,71)\), should provide an additional level of protection from immune surveillance at the initiation of infection in vivo.

1.2.2. Construction of Mutant Viruses Deleted for IE and Other Toxic Functions

The first generation of replication-defective mutant viruses consisted of mutants deleted for the essential ICP4 gene. One of these recombinants, designated d120, can be grown on a complementing cell line (E5) that expresses ICP4 \textit{in trans} on infection with the ICP4 deletion mutant virus \((35)\). We have subsequently introduced the \(\beta\)-galactosidase (lacZ) reporter gene, under control of the strong human cytomegalovirus (HCMV) immediate early gene promoter, into the thymidine kinase gene locus of the genome of this replication-defective mutant in order to rapidly identify cells and tissues infected with this vector to evaluate the potential uses for such vectors. We have shown that the transgene was transiently expressed in a variety of cell types in culture and in various tissues in vivo. Long-term expression of the transgene both in culture and in some cells in vivo may be affected by the virus-induced toxicity leading to death of the cells in culture and/or immune recognition and clearance in vivo. Therefore, it is clear that additional IE genes must be removed to further reduce viral toxicity.

In order to generate second generation replication-defective viruses deleted for multiple IE essential gene functions, it was necessary to construct a cell line to complement the essential IE gene functions. A cell line was constructed to complement both ICP4 and ICP27 by transfecting Vero cells with a plasmid containing the HSV-1 sequences coding for ICP4 and ICP27 along with a
neomycin-expression (SV2-neo) cassette for rapid selection of individual clones. In order to eliminate the chance of homologous recombination and rescue of the mutant viruses during propagation in the complementing line, the plasmid containing the coding regions for ICP4 and ICP27 was engineered to avoid overlap of these sequences with the deletions present within the virus. One clone, designated 7B, was isolated after multiple rounds of drug selection and this clone was able to complement the growth of the IE deletion mutants d120 (ICP4−) (35) and 5dl1.2 (ICP27−) (75).

A second generation, double-mutant virus deleted for both ICP4 and ICP22 was engineered using the 7B cell line (Fig. 3A). This recombinant, d4/22, was engineered by recombining the linearized plasmid PB5, containing the HCMV IE promoter-lacZ BGHpA cassette surrounded by ICP22 flanking sequences, into the ICP22 gene locus of the ICP4 replication-defective mutant d120. Positive recombinants were propagated and isolated on the 7B complementing cell line owing to the production of blue plaques following X-gal staining (Fig. 3B). The d4/22 recombinant fails to replicate or produce the blue plaque phenotype on normal noncomplementing (Vero) cells (Fig. 3B).

The cassette combined into the ICP22 locus is unique in that it contains the lacZ gene construct flanked by two 8-bp recognition sites for PaeI that are not present at any other site in the viral genome. Thus, the expression cassette can be easily removed by digestion with the PaeI restriction enzyme followed by religation of the genome yielding a recombinant with a single PaeI site that produces clear plaques on the 7B complementing cell line. In order to efficiently introduce a new gene cassette into the ICP22 locus of this recombinant, the recombinant genome is simply cleaved with PaeI and used in marker transfer transfection assays with plasmid-containing sequences that span the PaeI site in ICP22. The desired recombinant will lack the HCMV-lacZ expression cassette, and thus can be easily isolated as a clear plaque on a background of blue plaques produced by the parental virus. The recombination frequency obtained using this approach is tenfold greater than that seen in standard marker rescue experiments.

The d4/22 double mutant was examined for reduced toxicity in cell culture compared to both wild-type (KOS strain) and the d120 (ICP4−) parental virus. Myoblasts were infected with the various viruses at a multiplicity of infection (MOI) of 0.5 and the number of viable cells was determined at 2 and 4 d postinfection using trypan blue exclusion. This specific MOI was chosen since d120 uniformly kills cells at an MOI of one or greater. As shown in Fig. 4, this double mutant (d4/22) is less cytotoxic than d120 for myoblasts at an MOI of 0.5, and much less toxic than wild-type virus, although some toxicity still remains. It is believed that the removal of other toxic gene targets from the d4/22 double mutant should further reduce HSV vector toxicity to a point that it should eventually approach that observed with mock-infected cells.
Fig. 3. HSV vector mutation strategies and propagation. (A) Construction of ICP4−/ICP22− double deletion mutant. To construct the d4/22 mutant, d120 (ICP4−) viral DNA was transfected into the 7B (4/27) line along with the plasmid PB5 in which ICP22 sequences were deleted and replaced with the HCMV IE promoter-βgalactosidase BGH pA cassette. (B) The resulting recombinant can be isolated and propagated on the 7B line producing blue plaques because of the presence of the HCMV IEp-βgalactosidase BGHpA cassette in the ICP22 locus. No plaques are detected on noncomplementing Vero cells. In addition, the d4/22 mutant will also produce blue plaques on the E5 ICP4 complementing cell line (35).
Fig. 4. Cytotoxicity of recombinant viruses. Myoblasts (1.8 × 10^6) were either mock-infected or infected with wild-type KOS, DZ (ICP4⁻), or the d4/22 (ICP4⁻/ICP22⁻) double deletion mutant (MOI = 0.5). The number of viable cells were counted at 2 and 4 d postinfection. The increase in cell numbers observed in mock and other samples reflects myoblast cell division over the 2–4-d time period. Deletion of ICP22 from the ICP4 backbone reduced toxicity.

2. Materials

1. Vero (African green monkey kidney ATCC#CCL81) cells, other permissive cells such as HELs or BHKs, or complementing cell lines are required to propagate HSV-1 accessory or essential gene deletion viruses.

2. MEM-10%FCS: Eagle’s Modified Essential Medium supplemented with nonessential amino acids, 100 U/mL penicillin G, 100 µg/mL streptomycin sulfate, 2 mM glutamine, and 10% fetal calf serum.

3. TBS, pH 7.5: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA.

4. Lysis buffer: 10 mM Tris-HCl, pH 8.0, 10 mM EDTA.

5. TE: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.


7. 2X HBS: 20 mM HEPES, 135 mM NaCl, 5 mM KCl, 5.5 mM dextrose, 0.7 mM Na₂HPO₄, pH 7.05. Accurate pH of this solution is critical.

8. 1X MEM/X-gal/agarose overlay: Add an equal volume of 2X MEM-20% FCS containing 200 U/mL penicillin G, 200 µg/mL streptomycin sulfate, and 4 mM glutamine to an equal volume of 1.5% (w/v) low melting point (LMP) agarose that was autoclaved and cooled to 37°C and contains the chromogenic substrate X-gal at a final concentration of 300 µg/mL. X-gal is highly insoluble and must be dissolved in dimethyl formamide (DMF).
9. 1.0% Methylcellulose overlay: Add 25 g methylcellulose to 100 mL PBS, pH 7.5, in a 500-mL sterile bottle containing a stir bar. Autoclave the bottle on liquids cycle for 45 min. After the solution cools, add 350 mL of MEM supplemented with nonessential amino acids, 100 U/mL penicillin G, 100 µg/mL streptomycin sulfate, and 2 mM glutamine. Mix well and place the bottle on a stir plate at 4°C overnight. Once the methylcellulose has entered solution, add 50 mL of FCS.

10. 1X PBS, pH 7.5: 135 mM NaCl, 2.5 mM KCl, 1.5 mM KH₂PO₄, 8.0 mM Na₂HPO₄, pH 7.5.

3. Methods

3.1. Isolation of Viral DNA for Transfection

Engineering a new virus recombinant requires a plasmid containing the particular sequence of interest flanked by sufficient amounts of viral sequences homologous to the desired gene locus within the HSV-1 genome, along with purified, infectious viral DNA. The quality and purity of these two reagents will determine the frequency of generating the desired recombinant virus. It is imperative that the sequence of interest in the plasmid contain at least 500–1000 bp of flanking HSV-1 sequences to achieve a higher frequency of producing and isolating the recombinant. The quality of the viral DNA used in the transfections to synthesize new recombinants can be evaluated by two criteria: whether the DNA is intact and at the proper concentration, as determined by Southern blot analysis (76); or, more importantly, whether the viral DNA is infectious and capable of producing an optimal number of plaques following transfection of 1 µg of viral DNA. We have optimized the protocol for the production of highly infectious viral DNA and routinely obtain preps in which 1 µg of purified viral DNA will yield 100–1000 plaques.

1. Infect a subconfluent to confluent monolayer of cells in a T150 tissue culture flask at an MOI of 3. The cell should have been split at both 1 and 3 d prior to infection.
2. Allow the infection to proceed for approx 18–24 h depending on both the cell type and virus used. All cells should be rounded-up and still adhered to the flask, yet they should be just about ready to detach.
3. Remove the cells by tapping the flask or use a cell scraper to dislodge the cells.
4. Pellet the cells for 5–10 min at 2000–3000 rpm at 4°C in a 15-mL conical polypropylene tube.
5. Wash the cells once with 10 mL of TBS (pH 7.5).
6. Lyse the cells in 5 mL of lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA) plus 0.25 mg/mL proteinase K plus 0.6% SDS.
7. Wrap the lid of the tube in parafilm and incubate the tube on a nutator platform at 37°C overnight.
8. Extract the suspension twice with phenol:chloroform:isoamyl alcohol (25:24:1), being careful to not be too vigorous. Yet, it is important to invert the tube enough
to achieve proper mixing of the phases. When removing the aqueous phase, remember to leave the interface behind, as the DNA stays at this point, thus increasing the overall yield.

9. Extract the aqueous phase twice with chloroform, again being careful during mixing.

10. Remove the aqueous phase to a new tube, going as close to the interface as possible. The DNA present at the interface is extremely viscous and will enter the pipet as a visible slurry.

11. Add 2 vol of cold isopropanol to precipitate. Mix well.

12. The DNA can be spooled on a heat-sealed Pasteur pipet or the mixture can be stored at -20°C overnight. If spooling the DNA, remove the spooled DNA and transfer the pipet to a new tube, breaking off the Pasteur pipet. Let dry overnight, then resuspend in 0.5–1.0 mL TE buffer or dH2O using wide-bore pipet tips.

At this point, it is important to determine the quality of the DNA prep. The DNA concentration is determined spectrophotometrically at 260 nm. Southern blot analysis of digests of 1 μg quantities of the DNA prep with several diagnostic restriction enzymes should determine whether the DNA is intact. Transfection of 1 μg quantities of the DNA prep into permissive tissue culture cells will allow the determination of the number of infectious particles per 1 μg of DNA.

3.2. Construction of Recombinant Virus: Transfection of Plasmid/Viral DNA

Once the proper plasmid has been constructed, deleting the HSV-1 gene of interest yet maintaining sufficient HSV-1 flanking sequences to allow efficient recombination into the viral genome, it is possible to marker transfer these sequences into the virus taking advantage of the highly active recombinational machinery of the virus by transfecting both linearized plasmid and purified viral DNA into permissive cells using the calcium phosphate method (77,78). Cloning of a reporter gene cassette at the site of the deleted HSV-1 sequences in the plasmid will allow for a more rapid identification and purification of the desired recombinant virus from the ensuing transfection reaction.

1. Split Vero cells (for deletion of nonessential HSV-1 genes) or the specific complementing cell line (for deletion of essential HSV-1 gene[s]) 1.10 3 d prior to transfection.

2. At 1 d prior to transfecting, split the cells again and plate in 60-mm tissue culture dishes at a cell density of 1 x 10^6 cells/plate in MEM-10% FCS.

3. Linearize the plasmid construct at a restriction enzyme site between the flanking HSV-1 sequence and any Escherichia coli DNA present within the bacterial vector.

4. Make up the transfection mixture by adding 1–5 μg from the viral DNA prep (the amount of DNA that yields 200 or more plaques by transfection) to an amount of linearized plasmid DNA (or restriction fragment) equal to 10X and 50X genome equivalents of viral DNA. Viral DNA or plasmid DNA alone is used as controls for the transfection.
5. Add 600 μL of 2X HBS to each tube, mix, and place on ice for 20 min.
6. Add 41 μL of 2M CaCl₂ dropwise, mixing gently. Set at room temperature for 20 min.
7. Aspirate the media from the plates and rinse with 1 mL of 2X HBS.
8. Pipet transfection mixture up and down to break-up large clumps of precipitate, add carefully to cell monolayers, and place plates at 37°C for 40 min in a CO₂ incubator.
9. Add 4 mL MEM-10% FCS per plate and place at 37°C for 4 h in a CO₂ incubator.
10. Aspirate media from plates and wash with 1 mL of HBS, being careful not to cause the cell monolayer to lift off the plate.
11. Slowly and carefully add 2 mL of 20% glycerol (dilute 100% glycerol in 2X HBS) per plate and leave on cells at room temperature for exactly 4 min.
12. Carefully remove all of the glycerol shock solution by aspiration and wash the monolayer three times with 2 mL of MEM-10% FCS. Be sure that the monolayer remains intact.
13. Carefully add 4 mL of MEM-10% FCS, and incubate the plates at 37°C in a CO₂ incubator.
14. Observe the plates twice daily under the microscope for the production of CPE, indicating the presence of infectious foci. This usually takes 3–5 d depending on the virus and cell type used to propagate the recombinant.
15. Once a majority of the cells have rounded up, the media is removed and stored temporarily at 4°C, and virus is isolated from the cell pellet by three cycles of freeze-thawing and sonication followed by centrifugation at 2000 rpm for 5 min at 4°C, and this supernate is then combined with the removed media and stored at −80°C for use as a stock.

This stock of virus can now be analyzed for the presence of recombinants and purified as described in the next section.

### 3.3. Isolation and Purification of Recombinant Virus

The stock of virus obtained from the transfection can now be used to isolate the desired recombinant. If recombination of the sequences into the viral genome results in deletion of an essential HSV-1 gene product, then it is easy to screen for recombinants by simply replica plating virus on both the complementing cell line and on the noncomplementing line (Vero). The desired recombinant will only be capable of producing plaques on the complementing cell line. However, deletion of nonessential functions cannot be selected for and thus it is necessary to isolate and purify the recombinant through three rounds of limiting dilution. This process can be greatly enhanced by the inclusion of a reporter gene cassette within the sequences to be recombined back into the viral genome. We have optimized the limiting dilution procedure for the detection of recombinants expressing the β-galactosidase (lacZ) reporter gene.
3.3.1. Limiting Dilution Procedure for the Detection of Recombinants Expressing LacZ

The advantage of employing limiting dilution is that it does not require the standard plaque isolation technique in which single well-isolated plaques are picked following agarose or methylcellulose overlay. Contamination of positive recombinants with parental virus represents a considerable problem using the standard plaque isolation procedure since it is difficult to find well-isolated plaques on a plate, and virus from an adjacent plaque can get sucked up along with that from the designated plaque in the act of picking a plaque through the agarose or methylcellulose overlay.

1. Titer the stock of recombinant virus from the transfection.
2. Add 30 PFU of virus to 3 mL of $1 \times 10^6$ cells in suspension (MEM-10% FCS) within a 15-mL conical polypropylene tube.
3. Wrap the lid of the tube with parafilm, and place the tube on a nutator rocker platform at 37°C for 1 h.
4. Following the 1-h period for virus adsorption, add 7 mL of fresh media and plate 100 μL of the mixture in each well of a 96-well flat-bottomed plate.
5. Incubate the plates at 37°C in a CO₂ incubator for a period of 2–5 d, until the appearance of plaques.
6. The plates are scored for wells containing only single plaques. Theoretically, by adding 30 PFU, it should be possible to obtain 30 out of 96 wells that contain single plaques.
7. Transfer the media from each well into a new 96-well plate. Store the plate at −80°C for future use as a virus stock.
8. Overlay each well with 100 μL 1X MEM/X-gal/agarose using a multichannel pipetor.
9. Let the agarose overlay solidify at room temperature and then incubate the plates at 37°C in a CO₂ incubator overnight or for several hours until the appearance of readily detectable blue plaques occurs.

Wells containing single blue plaques are scored and the overall frequency of original recombination event is determined. Media from the frozen stock 96-well plate stored at −80°C can now be used in the next round of the limiting dilution procedure. With each subsequent round of limiting dilution, the ratio of desired recombinants within the population should approach 100%. We will routinely proceed through one additional round of limiting dilution after the ratio reaches 100% to ensure purity of the stock. At this point, the virus stock can be used to produce a midi-stock for the eventual preparation of a high titer stock for general experimental use and, at the same time, this stock is used to produce viral DNA to confirm the presence of the insert as well as the absence of the deleted sequences by Southern blot analysis (76).
3.3.2. Limiting Dilution for the Detection of Recombinants Lacking Reporter Genes

If the recombinant virus lacks a lacZ expression cassette, it is still possible to isolate and identify the desired recombinant using the limiting dilution technique in combination with either dot blot (79) or Southern blot (76) analyses. Steps 1–6 are identical to the above procedure.

1. Titer the stock of recombinant virus from the transfection.
2. Add 30 PFU of virus to 3 mL of $1 \times 10^6$ cells in suspension (MEM-10% FCS) within a 15-mL conical polypropylene tube.
3. Wrap the lid of the tube with parafilm, and place the tube on a nutator rocker platform at 37°C for 1 h.
4. Following the 1-h period for virus adsorption, add 7 mL of fresh media and plate 100 μL of the mixture in each well of a 96-well, flat-bottomed plate.
5. Incubate the plates at 37°C in a CO₂ incubator for a period of 2–5 d until the appearance of plaques occurs.
6. The plates are scored for wells containing only single plaques. Theoretically, by adding 30 PFU, it should be possible to obtain 30 out of 96 wells that contain single plaques.
7. Transfer the 80 μL of the media from each well into a new 96-well plate. Store the plate at −80°C for future use as a virus stock.
8. For each of the single plaque wells, use the remaining 20 μL of media to infect one well of a 24-well plate containing subconfluent-to-confluent cell monolayers.
9. Incubate the plates at 37°C in a CO₂ incubator for a period of 2–3 d until the appearance of virus-induced CPE is evidenced by cell rounding.
10. Scrape each well with a pipetman tip to remove the monolayer and transfer to a 1.5-mL microfuge tube.
11. Spin for 10 min in a microfuge at room temperature to pellet cells and virus.
12. Aspirate off the supernate and wash with 500 μL of TBS, pH 7.5.
13. Pellet the cells for 5 min in a microfuge, and aspirate off the supernate.
14. Resuspend the pellet in 200 μL of lysis buffer containing 25 mg/mL proteinase K and 0.6% SDS.
15. Digest overnight at 37°C on a nutator rocking platform or on an orbital shaker.
16. Extract the suspension with phenol:chloroform:isoamyl alcohol (25:24:1), vortexing vigorously to mix the two phases. It is not essential to be careful during mixing since it is not crucial for this prep of viral DNA to be highly infectious. Again, when removing the aqueous phase, remember to leave the interface behind.
17. Extract the aqueous phase with chloroform.
18. Transfer the aqueous phase to a new tube, going as close to the interface as possible. The DNA present at the interface is extremely viscous, and will enter the pipet as a visible slurry.
19. Add 500 μL of cold isopropanol to precipitate. Mix well and store overnight at −20°C.
20 Spin tubes in microfuge for 15 min and remove supernate by aspiration.
21. Wash pellet with 500 µL of 70% ethanol, spin tubes in microfuge for 5 min, remove supernate by aspiration, and air dry.
22. Resuspend pellet in 50 µL of TE or dH₂O.

At this point, the viral DNA can be used either in dot blot (79) or Southern blot (76) hybridization analyses employing probes specific to the inserted sequences. Southern blot analyses are more suited to identifying recombinants containing a deletion of viral sequences owing to the production of an altered restriction pattern with the recombinant.

3.4. **Preparation of High-Titer Stocks of Recombinant Virus**

Prepare a midi-stock of recombinant virus from a monolayer of cells in a T25 tissue-culture flask and obtain the titer of the stock for preparation of the final stock.

3.4.1. **Titration of Virus Stock**

This procedure can be used to obtain the titer of any size virus stock.

1. Seed six-well tissue culture plates with 0.5–1.0 × 10⁶ cells per well at 1 d prior to titration of the stock.
2. Prepare a series of tenfold dilutions (10⁻²–10⁻¹⁰) of the virus stock in 1 mL of cold MEM without serum.
3. Add 100 µL of each dilution to a near confluent monolayer of cells in a single well of a six-well tissue culture plate (in duplicate).
4. Allow the virus to adsorb for a period of 1 h at 37°C in a CO₂ incubator. Rock the plates every 15 min to distribute the inoculum.
5. Aspirate off the virus inoculum, add 3 mL of 1.0% methycellulose overlay and reincubate the plates for 3–5 d until well-defined plaques appear.
6. Aspirate off the methycellulose, and stain with 1 mL of 1% crystal violet solution (in 50:50 methanol dH₂O v/v) for 5 min. The stain fixes the cells and virus.
7. Aspirate off stain, rinse gently with tap water to remove excess stain, and air dry.
8. Count the number of plaques per well, determine the average for each dilution, and multiply by a factor of 10 to get the number of plaque forming units/mL (PFU/mL) for each dilution. Multiply this number by 10 to the power of the dilution to achieve the titer in PFU/mL.

3.4.2. **Virus Stock Preparation**

The following procedure calls for preparing a virus stock from two roller bottles worth of cells; however, it can either be scaled up or down depending on specific needs.

1. Seed two 850-cm² roller bottles with 2.0 × 10⁷ cells per roller bottle 1–2 d prior to infection.
2. Decant media from the bottles.
3. Add $1 \times 10^6$ PFU of virus (MOI = 0.01) in 10-mL of MEM without serum per roller bottle.
4. Allow the virus to adsorb for a period of 1 h at 37°C.
5. Remove the inoculum and add 100–125 mL MEM-10% FCS.
6. Incubate at 37°C until all the cells have rounded-up and are starting to come off the monolayer (2–3 d). It is important to observe the roller bottles at least twice daily under the microscope, as the exact time of harvest is crucial to obtaining a high-titer stock and depends on the virus and cell type used to propagate the recombinant.
7. Dislodge the cells from the plastic surface either by tapping the roller bottle or through the use of a cell scraper and transfer the suspension to 50-mL conical polypropylene tubes.
8. Pellet the infected cells at 2500 rpm for 5 min at 4°C.
9. Decant the supernate into new tubes and store temporarily at 4°C.
10. Resuspend the pellets into a final vol of 2 mL MEM-10% FCS.
11. Subject the infected cell suspension to three cycles of freeze-thawing (−80°C→37°C).
12. Sonicate the suspension of 5 s.
13. Pellet the cell debris at 3000 rpm for 5 min at 4°C.
14. Resuspend the pellet in 1 mL MEM-10% FCS and combine with the supernate stored at 4°C.
15. Transfer to 50-mL Oakridge polypropylene tubes and pellet the virus at 20,000 rpm for 30 min at 4°C.
16. Carefully aspirate of the supernate and resuspend the pellets into a final volume of 1–2 mL of MEM-10% FCS. Aliquot the virus into 2-mL cryotubes and store at −80°C.

The titer of the stock can be determined by the virus titration procedure detailed above. In order to further purify the virus, the virus pellets can be resuspended in PBS instead of MEM-10% FCS and centrifuged on sucrose, dextran T10, or nycodenz gradients. Following these various separation procedures, it is necessary to add glycerol to a final concentration of 10% to the virus stock in order to properly cryopreserve the virus.

4. Notes
1. The DNA that is produced using the viral DNA isolation technique is not composed exclusively of viral DNA, but also contains cellular DNA. The cellular DNA in this mixture acts as carrier when precipitating the DNA during the isolation, thus increasing the yield of DNA. In addition, the cellular DNA acts as carrier DNA during transfections and increases the overall efficiency of forming a precipitate, thereby increasing the chance of obtaining the desired recombinant. If necessary, pure viral DNA can be prepared from virus harvested solely from the media of infected cells or from virus particles that have been gradient purified. The yield of DNA obtained in this instance is significantly reduced.
2. The use of wide-boar pipetman tips (Bio-Rad) will help prevent shearing of the viral DNA, thereby increasing the infectivity of the viral DNA preparation.

3. The quality of the viral DNA preparation is crucial to the recombination frequency. The quality of the viral DNA can be evaluated by Southern blot or by determining the number of infectious centers following transfection. We have found that some preps will appear to be intact by Southern blot analysis, yet still contain a significant number of viral genomes that are nicked and, thus, are not infectious. It is important that the preparation yields 100–1000 plaques/µg of viral DNA.

4. The quality of the plasmid DNA also plays a factor in the recombination frequency. Between 2 and 500 bp of HSV-1 flanking sequence is sufficient; however, 1 kb or greater will dramatically increase the chance of isolating the desired recombinant. The size of the sequence to be inserted into the HSV-1 vector genome can affect the generation of the desired recombinant. It is possible for the virus to package up to an additional 10% of the genome, 15 kb for wild-type virus and potentially more for single and multiple gene deleted viruses. If the insert is too large or contains sequences that affect the stability of the viral genome, part or all of the insert will be lost over time and it will not be possible to obtain a purified isolate of the desired recombinant. The specific gene locus targeted for insertion/deletion can affect the recombination event. Recombination into the repeat sequences can yield a mixture of viruses containing insertion into one or both copies of the gene. The recombinational machinery of the virus can convert an isolate with a single copy into a recombinant with inserts in both copies. The same mechanism can also produce virus lacking the insert in both copies (i.e., wild-type virus). Southern blot analysis can confirm whether the insert is present in 0, 1, or 2 copies.

5. It is important to linearize the plasmid construct before transfection to increase the recombination frequency compared to that obtained with uncut supercoiled plasmid. Digestion of the plasmid to release the insert, followed by purification of the restriction fragment does not increase the recombination frequency. Although the frequency is the same, use of purified fragment is superior since no chance exists for the insertion of plasmid vector sequences into the virus by semihomologous recombination.

6. The pH of the HBS transfection buffer (HEPES) is extremely crucial to the transfection efficiency. Depending on the cell type being transfected, other buffers such as BBS (BES) or PIBS (PIPES) may result in higher efficiencies.

7. Other transfection procedures can also be employed, such as lipofectin, that will produce equivalent or greater recombination frequencies.

8. Glycerol or DMSO can be used to shock cells during transfection. The percentage of glycerol or DMSO used depends on the cell type being transfected. We have found that glycerol is less toxic than DMSO for Vero cells and that 20% glycerol produced the highest number of transformants with the lowest level of toxicity.

9. Blue-gal can be substituted for X-gal in the 1X MEM/agarose overlay. Although Blue-gal is more costly than X-gal, it is superior to X-gal since it produces a darker blue reaction product with reduced background staining of the cell monolayer and it has a greater solubility in DMF.
10. The viral DNA miniprep procedure routinely yields enough DNA for 3–5 restriction enzyme digestions for Southern blot analysis.

11. Virus stocks should be maintained at a low passage. Use one vial of a newly prepared stock as a stock for preparing all future stocks. In order to reduce the chance of rescuing wild-type virus during the propagation of viruses carrying deletions of essential gene(s), stocks should be routinely prepared from single plaque isolates.

References


    simplex virus transcript abundant in latently infected neurons is dispensable for
    establishment of the latent state Virology 166, 254–257
14. Leib, D. A., Bogard, C. L., Kosz-Vnenchak, M., Hicks, K. A., Coen, D. M., Knipe,
    transcript of herpes simplex virus type 1 reactivates from the latent infection. J
    Virol 63, 2893–2900
    plex virus type 1 latency-associated transcript plays no role in establishment or
16. Steiner, I., Spivack, J. G., Lirette, R. P., Brown, S. M., MacLean, A. R., Subak-
    Sharpe, J., and Fraser, N. W. (1989) Herpes simplex virus type 1 latency-assoc-
    iated transcripts are evidently not essential for latent infection. EMBO J 8, 505–511
17. Roizman, B. and Sears, A. E. (1990) Herpes simplex viruses and their replication,
    in Field’s Virology, 2nd ed. (Fields, B. N., Knipe, D. M., Chanock, R. M., Hirsch,
    M. S., Melnick, J. L., and Roizman, B., eds.), Raven, New York, pp 1795–1841
    a function(s) that destabilizes both host and viral mRNAs. Proc Natl Acad. Sci.
    USA 84, 1926–1930
    virion-associated shut off of host polypeptide synthesis and exhibiting abnormal
    Construction and characterization of a herpes simplex virus type 1 mutant unable
    of herpes simplex virus DNA sequences which encode a trans-acting polypeptide
    required for virion-mediated induction of herpes simplex virus type 1 α genes
    Proc Natl Acad Sci USA 84, 71–75.
    protein mediating α gene induction in herpes simplex virus type 1-infected cells


Methods for the Use of Poliovirus Vectors for Gene Delivery

Casey D. Morrow, David C. Ansardi, and Donna C. Porter

1. Introduction

Poliovirus is a member of the Picornaviridae family of viruses. Characteristic of all members of this family, the poliovirus genome consists of approx 7500 bp of RNA of the plus sense polarity (1,2). Poliovirus is undoubtedly one of the most thoroughly characterized animal viruses. The three-dimensional structure of the entire virion is known (3), an infectious cDNA clone of the poliovirus genome has been generated (4,5), the entire nucleic acid sequence of poliovirus has been determined (1), and the cellular receptor that poliovirus uses to enter cells has been cloned and sequenced (6). The availability of a transgenic mouse expressing the poliovirus receptor has facilitated further description of the pathogenesis of poliovirus (7,8).

The expression of the poliovirus genome occurs by the translation of the entire open reading frame, resulting in a long polyprotein that is subsequently processed by virus-encoded proteinases 2A and 3C (9) (Fig. 1). The initial proteolytic processing results in the release of a capsid precursor protein, P1, by the proteinase 2A (10). The subsequent processing of the nonstructural precursor proteins P2 and P3 is accomplished by proteinase 3C (11). The capsid precursor P1 is efficiently processed by 3C\textsuperscript{pro} in the form of a fusion protein between 3C\textsuperscript{pro} and 3D\textsuperscript{pol}, called 3CD (12). Previous studies have established that processing of P1 by 3CD will occur efficiently even when the two proteins are expressed from individual recombinant vaccinia viruses (12,13).

The availability of an infectious clone of poliovirus has provided an opportunity to utilize molecular biologic techniques to investigate various regions of the poliovirus genome that are required for replication. Early studies have demonstrated that the majority of the P1 region of poliovirus could be deleted as
Fig. 1. Genomic organization of poliovirus: The entire poliovirus genome has been cloned and the 7500 bp nucleic acid sequence determined (1). The viral RNA genome contains a long 5' nontranslated region (approx 750 nucleotides containing an internal ribosome entry site [IRES]). Translation begins at nucleotide 743 and results in the synthesis of a long polyprotein. The amino-terminal glycine of the polyprotein is covalently linked to a single molecule of a 14-carbon fatty acid, myristic acid (a tetradecanoic acid). The processing of the polyprotein occurs by two viral-encoded proteinases, 2A and 3C. Following translation of the 2A proteinase, the autocatalytic activity of this enzyme releases the capsid precursor P1 from the polyprotein (10). The capsid precursor is subsequently processed by the 3C proteinase in the form of a precursor, 3CD, to give the viral capsid proteins VP0, VP3, and VP1. Following encapsidation of the viral genomic RNA, VP0 is further processed by an as yet unknown mechanism, giving VP4 and VP2. Further processing of the P2 and P3 region polyproteins is carried out by the viral 3C proteinase. Processing of the P3 polyprotein results in the release of a viral-genome linked protein, VPg, which is found attached to all 5' poliovirus RNA molecules, and is believed to be involved in replication and encapsidation of genomic RNA. The viral RNA-dependent RNA polymerase, 3D, is released from the polyprotein by the catalytic activity of 3C. Further details regarding the sequential cleavage of the P2 and P3 proteins and the kinetics of this reaction can be found in Lawson and Semler (29). The processing of the P1 polyprotein by the 3CD viral proteinase has been demonstrated to occur in trans using both in vitro and in vivo systems (12,13).
Fig. 2. Schematic of a poliovirus replicon: In previous studies, we have described poliovirus replicons that express regions of the HIV-1 genome (17,19,20). We have found that we can substitute foreign gene sequences between nucleotides 949–3359 of the poliovirus genome. This allows us to substitute a sequence of approx 2.5 kb of genetic information to maintain the appropriate size of the poliovirus genome. The plasmid containing the poliovirus replicon is linearized using the SalI restriction endonuclease. Following in vitro transcription using T7 RNA polymerase, the RNA is transfected into cells. The replication of the RNA genome results in the synthesis of a polyprotein containing a VP4-foreign gene fusion protein that can be detected in the transfected cells using appropriate antibodies. Synthetic proteolytic cleavage sites for the 2A proteinase is engineered at the amino and carboxy termini of the foreign gene to express proteins that can be subsequently released from the poliovirus polyprotein (20).

long as the translational reading frame was maintained between the P2 and P3 regions (14–16). Replication of a chimeric genome in which foreign gene sequences were inserted into the poliovirus genome were observed as long as the translational reading frame was maintained between the inserted genes and the remainder of the poliovirus polyprotein (17,18). These chimeric genomes maintain the capacity for replication and are referred to as “replicons.” To date, however, there has been no demonstration that genomes that do not have the capacity to replicate (i.e., in which the translational reading frame is disrupted) can be complemented in trans by viral proteins. Previous studies from our laboratory have demonstrated that between 800 and 2600 bp of foreign gene sequences can be substituted in the poliovirus genome (17,19–21). A prototype replicon is presented in Fig. 2. In this replicon, the foreign gene is positioned between the VP4 coding sequences and the 2A gene of poliovirus. The foreign gene is positioned such that the translational reading frame is maintained between the VP4-foreign protein 2A protein. We have constructed these replicons such that cleavage sites for the 2A proteinase are positioned at the amino and carboxy termini of the foreign protein. The expression of the foreign protein occurs upon proteolytic processing of the VP4-foreign protein fusion protein from the remainder of the polyprotein, as a result of the autocatalytic activity of the 2A proteinase. A second cleavage occurs by the 2A proteinase in trans.
to release the foreign protein, which contains only minimal changes at the amino terminus (usually only two amino acid changes) and eight additional amino acids at the carboxy terminus (20). We have used this vector to express a number of different proteins, most of which retain their native features.

The poliovirus replicon cDNA is positioned immediately downstream from the phage T7 promoter. The plasmid templates are first linearized at a unique SalI restriction site outside of the viral genome; the DNA templates are then used for in vitro transcription to generate an RNA copy. The transfection of the replicon RNA into tissue culture cells results in the replication of the RNA. The replication features for these genomes are similar to those seen in poliovirus-infected cells in that there is an asymmetric overproduction of the plus strand RNA. Since the poliovirus replicons do not encode a functional P1 capsid precursor protein, the genomes do not have the capacity to spread to other cells. Previous studies from this laboratory have demonstrated that poliovirus genomes containing deletions in the P1 region, or replicons that contain foreign gene insertions in the P1 region, could be encapsidated into poliovirions if transfected into cells previously infected with a vaccinia virus (VV-P1), which expresses the P1 protein of poliovirus (19–22). The features of this complementation system are presented in Fig. 3. Upon transfection of the replicon RNA into cells previously infected with VV-P1, the replicon undergoes RNA replication and expression of viral proteins. Previous studies from this laboratory have demonstrated that transfection of poliovirus replicons into vaccinia virus-infected cells actually results in enhanced replication and expression of viral proteins (23). Expression of the viral proteins from the chimeric genome results in the production of 3CD proteinase, which processes the P1 protein expressed by the recombinant vaccinia virus in trans. Upon processing of the P1 protein, assembly of subviral intermediates of poliovirus as well as encapsidation of the chimeric RNA genome occurs. Amplification of the encapsidated RNA genomes is accomplished by reinfection of cells with the encapsidated replicons and VV-P1. Multiple serial passage of the encapsidated replicon in the presence of VV-P1 results in the production of high titer stocks of the encapsidated replicons. Removal of the vaccinia virus is accomplished using a combination of detergents and immunoabsorption with anti-vaccinia virus antibodies. The methods for production of the replicons, transfection into cells, and the generation of stocks of encapsidated replicons are described in the following sections.

2. Materials

All general laboratory chemicals were purchased from Sigma (St. Louis, MO). Restriction enzymes were obtained from New England Biolabs (Beverly, MA). Tissue culture media and supplements were purchased from Gibco-BRL.
Encapsidation of poliovirus replicons using VV-P1: Schematic representation of the encapsidation of the replicons transfected into cells previously infected with a recombinant vaccinia virus VV-P1, which expresses the poliovirus capsid precursor protein. This system is based on the ability of the poliovirus capsid precursor protein, P1, to be processed by the poliovirus protease 3CD, provided in trans from the replication of the replicon RNA. Once the P1 protein has been processed, the encapsidation of the replicon RNA ensues, resulting in a polio virion containing the encapsidated replicon.
(Gaithersburg, MD). The [35S] Translabel (methionine/cysteine) and methionine/cysteine-free DMEM were purchased from ICN Biochemicals (Costa Mesa, CA). The T7 RNA polymerase was prepared in this laboratory by the method of Grodberg and Dunn (24); alternatively, the transcription kit Megascript from Ambion (Austin, TX) can be used.

2.7. Tissue Culture Cells and Viruses

Two common cell lines, HeLa and BSC40 (a derivative of CV-1) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum (complete medium). The recombinant vaccinia virus VV-P1, which expresses the poliovirus P1 capsid precursor protein, has been previously described (13).

3. Methods

3.1. Overview

The first step in the expression of a foreign protein from a poliovirus replicon is the subcloning of the gene of interest into a modified poliovirus cDNA. We have fused foreign genes between nucleotides 949 and 3359 of the poliovirus genome. The gene is fused such that a synthetic 2A (tyrosine-glycine) cleavage site is positioned at the junction between the VP4 gene (amino terminus) and the foreign protein, which also has a 2A cleavage site at the carboxy terminus. We have found that following the expression of this fusion protein, the viral proteinase 2Apro will process the synthetic 2A positioned between VP4 and the foreign protein, respectively, resulting in the full-length desired protein. The processed protein will then need to be evaluated to determine if it has maintained its native conformation (20) (see Fig. 2). Standard molecular biology techniques can be used to subclone the foreign gene of interest into the replicon cDNA; to facilitate cloning, we have constructed replicon cDNAs that contain unique XhoI or SacI (5’) and SnaB1 (3’) restriction sites. In our previous studies, we used DNA oligomers and PCR to create the appropriate restriction sites in the foreign genes (20,21). Once a suitable replicon has been constructed, the following procedures should be carried out to obtain stocks of encapsidated replicons:

3.2. Preparations of Template and In Vitro Transcription

The preparation of the template replicon DNA required for in vitro transcription is a critical step. Single, well-isolated E. coli colonies transformed with replicon DNA are picked from the plate and inoculated directly into 200 mL of LB plus 50 mg/mL of ampicillin. After overnight growth, the E. coli are collected by centrifugation and processed using the alkaline lysis procedure (25). The plasmid DNA is purified by ultracentrifugation in CsCl2 gradients (25). After centrifugation, the band containing the DNA is visualized by UV
Poliovirus Vectors

and removed using a syringe. The ethidium is extracted from the DNA with water-saturated butanol and the DNA is dialyzed against TE buffer (10 mM Tris pH 7.8, 5 mM EDTA) overnight. The DNA is then precipitated with 0.3M NaOAC and 3 vol of 100% ethanol at -70°C overnight. The precipitate is collected by centrifugation, washed with 70% ETOH, dried, and resuspended in TE buffer; the DNA is quantitated by UV 260/280.

The DNA (5 μg) is linearized by SalI digestion, followed by successive phenol-chloroform (1:1) and chloroform extractions. The DNA is precipitated with 0.3M NaOAC and 3 vol of 100% ethanol at -70°C for at least 2 h. The precipitate is collected by centrifugation, washed once with 70% ethanol, and dried. The in vitro transcription mixtures (100 μL) contain 5 μg of linearized DNA template, 5X transcription buffer (100 mM Tris pH 7.7, 50 mM MgCl₂, 20 mM spermidine, 250 mM NaCl), 10 mM dithiothreitol, 2 mM each of GTP, UTP, ATP, and CTP, 40 U of recombinant RNasin (Promega, Madison, WI), and approx 5 μg of purified T7 RNA polymerase per reaction mixture. After 90 min at 37°C, 5% of the in vitro synthesized RNA was analyzed by native agarose gel electrophoresis. Under these conditions, the full-length RNA transcripts (approx 7.5 kb) comigrate with the 2.0–2.2 kb DNA markers obtained from HindIII digest of phage λ. We have found that the Megascript kit from Ambion works well for in vitro transcription of the replicon DNAs.

The in vitro transcribed RNAs can be transfected immediately or frozen at -70°C overnight prior to use. The best results are obtained if the in vitro synthesized RNA is transfected without further processing (such as phenol-chloroform extraction).

3.3. Transfection of replicon RNA

The transfection of the replicon RNA into cells can be accomplished using a variety of established methods. In our laboratory, we have utilized DEAE-Dextran as a facilitator (mol wt 500 kDa, obtained from Sigma). For our transfections, HeLa cells were first infected with 10 plaque-forming units (PFU) per cell of VV-P1. The amounts of virus and RNA used have been optimized for a single well of a six-well plate (Falcon, Los Angeles, CA, 3046). After 2 h of infection, the cells were transfected using a protocol adapted from the originally described method of Vaheri and Paganò (26). The cells are washed twice with phosphate buffered saline (PBS; calcium and magnesium-free). The cells are then incubated with approx 10 μg of the in vitro synthesized RNA (approximately one-fifth of the in vitro reaction) with DEAE-Dextran at a concentration of 300 μg/mL in PBS at 37°C for approx 2 h. Although the cells have a tendency to round up from this procedure, they do not detach from the plate. Following two h incubation, cells are washed carefully with DMEM, followed by the addition of complete medium.
3.4. Serial Passage of Encapsidated Replicons

Transfection of the replicon RNA into cells previously infected with VV-P1 results in expression of the appropriate poliovirus proteins (e.g., 3CD) for the proteolytic processing of the P1 protein expressed from VV-P1 (19–22). This results in the assembly of subviral particles and the subsequent encapsidation of the poliovirus replicon RNA. The procedure for passaging the encapsidated replicons is presented in Fig. 4. Twenty-four hours after transfection of the replicon RNA into VV-P1-infected cells, the cells are lysed on ice by the addition of Triton-X-100 at a final concentration of 1%. The lysates are then treated with RNase A for 10 min at room temperature, and the extract is clarified by low-speed centrifugation at 14,000g for 20 min. The supernatants are then adjusted to 0.25% SDS, and overlaid onto a 30% sucrose cushion (30% sucrose, 30 mM Tris pH 8.0, 1% Triton X-100, and 0.1M NaCl). Following centrifugation in a Beckman SW-55 Ti rotor at 290,000g for 1.5 h at 4°C, the supernatant is gently decanted and the pelleted virions are washed with a buffer consisting of 30 mM Tris pH 8.0, 0.1M NaCl. To avoid losses during the washing procedure, the pellet is again recentrifuged in the wash buffer for an additional 1.5 h using the Beckman SW 55 Ti rotor at 290,000g. The pellets are then resuspended in complete medium and designated as passage one of the replicons. For additional serial passage of the encapsidated replicons, BSC40 cells are first infected with 20 PFU/cell of VV-P1. At 2 h postinfection, the cells are infected with passage one of the encapsidated replicons. At this stage, care is needed to maintain the small volumes during the infection owing to the low titer of the encapsidated replicons. After 24 h, the infection is terminated by three successive freeze-thaws, followed by sonication and clarification by a low-speed centrifugation at 14,000g for 20 min. Previous studies from this laboratory have determined that the low-speed centrifugation results in the removal of approx 90–95% of the vaccinia virus from the cell lysate (19–22). The passage procedure is repeated by first infecting BSC40 cells with VV-P1, followed by reinfection with the cell supernatant. After 5–6 serial passages, the cell monolayer takes on a cytopathic effect consistent with that of a poliovirus infection rather than a vaccinia virus infection. This is easily visualized because poliovirus results in cellular destruction leading to the detachment of the cell monolayer from the culture flask, whereas vaccinia virus-infected cells do not readily detach. Once this type of cytopathic effect is observed, the stock of the encapsidated replicons can be increased by infecting larger amounts of BSC40 cells. Generally, this procedure is initiated by increasing the size from one well of a 24-well plate to two and then four wells of a 24-well plate, followed by one well of a 6-well plate, leading eventually to the serial passage of stock in large tissue culture flasks.
Fig. 4. Replicon encapsidation and passaging protocol: The procedure for the serial passage and development of stocks of the encapsidated replicon is presented. The important feature of this system is that, once the replicon has been successfully encapsidated, it can be passaged in a manner similar to poliovirus as long as coinfecting VV-P1 is present. Once sufficient stocks of the replicon have been obtained, removal of VV-P1 by either detergent treatment or immunoadsorption results in a pure stock of the encapsidated replicons.

3.5. Detection of Encapsidated Replicons

The detection of the encapsidated replicons can be accomplished by several methods. One method is to utilize radioactive probes specific for the inserted foreign gene sequence in the poliovirus genome. A simple dot blot procedure
can be used to detect the presence of the replicons (see Section 3.7.). A second method is to use metabolic labeling followed by immunoprecipitation to assay for protein expression from the encapsidated replicon. In this case, we use antibodies specific for the foreign protein to be expressed or antibodies to the poliovirus 3D polymerase, if antibodies to the foreign protein are not available. It is important to note that because the poliovirus RNA is translated into a single polyprotein that is then processed, a feature of the expression of foreign proteins using the replicons is that equivalent amounts of the foreign protein and the poliovirus replicative proteins, such as the 3Dpol (or 3CD) protein, should be expressed. To metabolically label cells, the cells are infected with the stocks of encapsidated replicons. At 6 h postinfection, the cells are starved for methionine-cysteine by incubation in DMEM minus methionine-cysteine for 30 min. After starvation, 100 μCi of 35S-Translabel is added to these cultures for an additional 1 h. Cultures are then processed for immunoprecipitation of viral proteins by lysing the cells with a buffer consisting of 150 mM NaCl, 10 mM Tris-pH 8.0, 1% Triton X-100, 1% sodium deoxychlate, and 0.2% sodium dodecyl sulfate (SDS, RIPA buffer). Upon lysis of the cells, the extract is centrifuged at 14,000g for 10 min to pellet any residual debris and the designated antibodies are added to the supernatants followed by incubation at 4°C for 24 h rocking. The immunoprecipitates are collected by addition of 100 μL of protein A Sepharose (10% wt/vol in RIPA buffer). After 1 h rocking at room temperature, the protein A Sepharose beads are collected by a brief centrifugation, washed four times with RIPA buffer, and the bound material is diluted by boiling for 5 min in gel sample buffer. The proteins can then be analyzed by SDS-PAGE; after fluorography the proteins can be visualized by autoradiography. In all cases thus far examined, we have found that the expression of the viral proteins correlates with the capacity of the RNA to replicate in the infected cells.

3.6. Isolation of Encapsidated Poliovirus Replicons Devoid of Vaccinia Virus

Once a sufficient stock of the encapsidated replicons is obtained, it is important to concentrate these replicons and remove any residual vaccinia virus. This can be accomplished by adjusting the concentration of the supernatant to 1% Triton X-100 and 0.25% SDS. The virus stock is then overlaid on a 30% sucrose cushion (30% sucrose, 30 mM Tris pH 8.0, 1% Triton X-100, and 0.1M NaCl), and the encapsidated replicons are pelleted through the cushion in an SW 41 rotor at 160,000g for 3 h at 4°C. Although the majority of the vaccinia virus has been inactivated by the detergents, a residual amount of vaccinia virus, generally less than 0.1% of the input amount, is still detectable in the sample. To remove the residual vaccinia virus, the pelleted virions are resuspended in PBS (phos-
phate-buffered saline) in the presence of antivaccinia antibodies (Lee Biomolecular, San Diego, CA). The sample is incubated at 4°C for 24 h, followed by the addition of protein A Sepharose. The protein A Sepharose is collected by low-speed centrifugation and the supernatant containing the encapsidated replicon is then used for further characterization. We have found that this procedure virtually eliminates any remaining vaccinia virus from the preparations.

3.7. Estimation of the Titer of Encapsidated Poliovirus Replicons

Since these replicons have the capacity to infect a cell but lack capsid proteins, the replicons cannot form plaques, and therefore virus titers cannot be quantitated by traditional assay. To overcome this problem, we have devised a method to estimate the titer of the encapsidated replicons by comparison with wild-type poliovirus of known titer. For these studies, RNA from a titered stock of poliovirus is isolated according to previously described procedures (27). The virion RNA is then precipitated in 2.5 vol of ethanol using 0.2M LiCl2. The encapsidated replicon RNA is extracted using the same procedure. Following precipitation, the RNAs are washed consecutively with 80% ethanol and 100% ethanol, dried, resuspended in DEPC-treated H2O, and serially diluted and spotted onto nitrocellulose paper (dot blot apparatus from Bio-Rad, Hercules, CA).

For detection of poliovirus-specific RNA, a 503-bp riboprobe was generated complementary to nucleotides 671–1174 of the poliovirus genome. The details for the construction of the plasmid to generate this riboprobe can be found in Choi et al. (17). The in vitro transcription of this plasmid results in a complementary RNA that will hybridize to the plus strand RNA of both wild-type poliovirus and the encapsidated chimeric RNA genomes. The in vitro transcriptions are performed under standard conditions using 5X transcription buffer (100 mM Tris pH 7.7, 50 mM MgCl2, 20 mM spermidine, 250 mM NaCl), 10 mM dithiothreitol, 2 mM each of ATP, GTP, and CTP, 32[P]-UTP, 40 U recombinant RNasin (Promega), and approx 5 µg of purified T7 RNA polymerase per reaction. The conditions for hybridization can be found in Choi et al. (17). To determine the relative titer of the encapsidated replicons, the radioactivity associated with each dilution was quantitated using a phosphorimager (or similar radiation scanner). A standard curve was generated in which the radioactive intensity is plotted vs the plaque-forming units of the wild-type poliovirus genome. Extrapolation of the radioactivity of the encapsidated replicon samples to the curve gives an estimation of the approximate titer of the stocks of encapsidated replicons. The resulting titer is expressed in infectious units of replicons, since the infection of cells with the replicons does not lead to plaque formation owing to the absence of the P1 capsid gene. We have determined experimentally that the infectivity of equal
amounts of infectious units of the encapsidated replicons correlates with similar amounts of plaque-forming units of wild-type poliovirus.

3.8. Future Directions

We have effectively encapsidated genomes that express a foreign gene of approx 3.0 kb (e.g., β-galactosidase). We have grown such replicons to titers of $10^7$ infectious U/mL using standard laboratory conditions. Recent studies from other laboratories have described the use of internal ribosome entry sites (IRES elements) positioned within the poliovirus genome to construct polycistronic replicons (28). This technology could easily be applied to these replicons to produce two different proteins from the same replicon. The design of these constructs would be limited only by the upper limit for the encapsidation of the poliovirus RNA genome, which is probably around 8000 bp (400–500 bp larger than the wild-type genome).

Finally, one of the unique features of these encapsidated replicons is that it is now possible to use these replicons as a means to deliver foreign antigens or biologically active proteins to cells in the absence of an infectious virus. Once suitable stocks of the encapsidated replicons have been generated, the complementing vaccinia virus, VV-P1, can be effectively removed, resulting in a stock of encapsidated replicons that have the capacity to infect a cell and undergo one round of replication. Since these replicons do not encode a functional capsid protein, they can be viewed as noninfectious, thus greatly reducing concerns of disease caused by the vector used in delivery of the foreign gene.

4. Notes

One of the critical features for the initiation and maintenance of the encapsidated genomes is to accurately titer the recombinant vaccinia virus expressing the poliovirus capsid precursor protein (VV-P1). It is important to titer on the particular cell type that will be used for the transfection and encapsidation process (i.e., HeLa or BSC40 cells). We have found that it is critical to maintain a multiplicity of infection of approx 20 PFU/cell so that every cell is infected. Great care has to be maintained during the passaging of the encapsidated genomes. In the early stages of passaging, there is a low level of encapsidated genomes and it is imperative that low culture volumes are maintained to increase the multiplicity of infection of the encapsidated genomes. Since the inherent infectivity of poliovirus is somewhat low (approx 40–1000 particles per infectious units), it is important at early stages to optimize the conditions for infectivity. As the number of serial passages increases, the levels of encapsidated replicons increase, allowing for the expansion of the stock of the encapsidated replicons. Again, during the expansion, care has to be taken to avoid overdilution of the encapsidated replicons.
Acknowledgments

We thank Dee Martin for preparation of the manuscript. This work is supported in part by NIH grants AI25005, AI15128, AI28147, DAMD 17-94-J-4403 (US Army), and a grant from the American Foundation for AIDS Research (AmFAR 50449-15-PG) to CDM.

References

Methods for the Construction of Human Papillomavirus Vectors

Saleem A. Khan and Francis M. Sverdrup

1. Introduction

A number of vector systems have been developed for the delivery of therapeutic genes into cells (1). Many viral vectors suffer from the disadvantage of random integration into the chromosome, making the expression of the cloned genes dependent on the chromosomal context of the inserted DNA. Papillomaviruses (PVs) are potentially important vector systems because of their extrachromosomal replication in target cells. The PVs are small DNA viruses that infect humans and a wide range of animals. Human papillomaviruses (HPVs) induce benign proliferative squamous epithelial and fibroepithelial lesions (warts and papillomas) in their natural hosts (2). Some HPVs are also involved in the pathogenesis of anogenital cancer and, in particular, cancer of the cervix (2). Papillomaviruses contain circular, double-stranded DNA of approx 8 kb, and usually replicate extrachromosomally at a copy number estimated to be between 10 and 100 (3). The potential advantages of PV vectors include expression of cloned genes from an extrachromosomal state that may be more amenable to uniform expression and possible elimination of problems associated with integration of DNA into transcriptionally inactive regions of the cellular chromosomes. Since PV DNA is not encapsidated, it may be possible to insert larger DNA sequences into such vectors, provided the DNA can still replicate in a stable manner. Bovine papillomavirus type 1 (BPV-1) vectors have been used to produce stable cell lines expressing foreign proteins (for reviews, see refs. 4–7). The BPV-1 vectors used in these studies contained both replication and transforming genes, and in most cases, extrachromosomal replication of these vectors was accompanied by transformation of the target cells (8–12). Recent advances in our understanding of the replication and transforming
genes of PVs have resulted in renewed efforts to develop vectors that can be established as stable extrachromosomal plasmids and express foreign proteins without oncogenic transformation of the host cell. The current article deals mostly with the potential of HPVs as vectors for gene therapy.

1.1. Organization of the HPV Genome

Most PVs have a similar genomic organization and their genome has been divided into the early and late regions. The early region includes eight ORFs (E1 to E8) involved in viral DNA replication, episomal maintenance, and cellular transformation (5). The late region comprises genes that encode the viral structural proteins, L1 and L2 (5). The E6 and E7 proteins of the high risk HPVs such as types 16 and 18 are involved in cell proliferation and tumor development (2,13). A number of recent studies with PVs have shown the requirement for the viral E1 and E2 proteins in DNA replication (14–19). The long control region (LCR) of PVs contains the promoter and enhancer sequences, including the binding sites for many viral and cellular factors. The LCR also contains the origin of replication (ori) of PVs (3,18,19).

1.2. Replication of Human Papillomaviruses

The keratinocytes of stratified squamous epithelia are the natural hosts for HPVs. In benign papillomas and condylomas, HPV DNA replicates as a multicopy plasmid. The copy number of HPV is influenced by the differentiation state of the cell. In the basal cells of squamous epithelium, HPV DNA replicates at a low but stable copy number, whereas in the upper differentiating cells, vegetative replication occurs resulting in a high copy number. In the final stages of terminal differentiation, the late genes encoding structural proteins L1 and L2 are expressed. HPVs such as type 1a that cause common warts produce large amounts of virion and DNA, whereas types 16 and 18, which are predominantly associated with anogenital cancers, produce extremely low levels of DNA and virions. It is important to note that vegetative replication of HPV DNA is not required for their potential use as vectors for gene therapy. The PVs have been extensively studied in recent years for their replication properties. These studies have shown that the viral E1 and E2 proteins are both necessary and sufficient to support the transient replication of plasmids containing the ori of PVs (14–19). In the case of HPV-1a, the E1 protein alone is sufficient for replication of ori-plasmids (20). The ori of PVs contains an E1 binding site and multiple E2 binding sites. The E2 binding sites appear to act cooperatively to increase the efficiency of replication. We have also found that a region upstream of the HPV-1a origin appears to negatively regulate replication in transient assays (20). The E1 protein of PVs is a nuclear phosphoprotein of approx 600 amino acids that has origin-binding, DNA helicase and ori-spe-
specific unwinding activities (21,22). Recently, the overexpression of the BPV-1 E1 protein has been shown to perturb the host cell cycle, resulting in an increase in the duration of the G2 and M phases (23). The viral E2 protein is an activator as well as repressor of viral transcription (24). The E2 ORF encodes several proteins that originate from selective promoter usage and alternative mRNA splicing. The full-length transactivator form of E2 activates transcription by binding to viral promoters located near the E2 binding sites. It also negatively regulates the transcription of the E6 and E7 oncogenes (24). The E2 protein stimulates HPV replication by forming a complex with E1 and enhancing its binding to the origin (25,26). To be useful for gene therapy, it is important to develop PV vectors that replicate extrachromosomally and do not cause oncogenic transformation of the target cells. This may be possible since recent studies have shown that, whereas the E1 and E2 genes are required for PV replication, the E6 and E7 oncogenes are dispensable at least for transient replication. Here we describe our recent efforts to develop HPV-based vectors and their replication in various cell lines.

1.3. Development of HPV Vectors

In order to identify viral sequences required for HPV replication, we have carried out transient replication analysis in various human cell lines. Plasmids containing the HPV-18 origin replicated efficiently in human C-33A, 293, HeLa, and IB-3 cell lines upon cotransfection with plasmids expressing the viral E1 and E2 proteins (19). The copy number of the HPV-18 ori plasmids was ~500–1000/cell. The above information was used to generate vectors in which all the elements required for replication (the E1 and E2 genes, and the origin) were present on a single plasmid (Fig. 1). In these constructs, the E1 and E2 proteins were expressed from various promoters. Although all HPV-based plasmids replicated to various extent in C-33A cells, plasmids pFS101 and pFS106 showed maximal replication. Plasmid pFS101 replicates to much higher levels than pFS102. The E2 gene in these two plasmids is expressed from the RSV 3' LTR and the HPV-18 LCR promoters, respectively. It is possible that high levels of E2 expressed from the stronger RSV promoter in pFS102 may inhibit DNA replication. This is consistent with previous studies in which high levels of E2 appeared to inhibit DNA replication (19,26). The copy number of pFS101 and pFS106 in transiently transfected cells was found to be approx 100/cell. Some of the plasmids shown in Fig. 1 were also found to replicate in IB-3 cells. We are now testing the stable replication of plasmids pFS101 and pFS102 that contain the hygromycin resistance gene in various cell lines. Additional HPV-18 vectors are being developed in which the E1 and E2 proteins are being expressed from various promoters in order to obtain vectors with a range of copy numbers.
We are also developing HPV-1a based vectors for gene delivery. It is well known that during in vivo infections, HPV-1a produces large amounts of virions and DNA compared to HPV-18. Transient replication of HPV-1a usually gives several-fold higher levels of replication than HPV-18. Replication analysis with HPV-1a showed that the E1 protein was sufficient to support replication of plasmids containing the HPV-1a origin (20). We have also found that an E1 binding site is sufficient to function as origin, and multimerization of the E1 binding site greatly stimulates replication. HPV-1a-based vectors are being developed that contain the E1 and E2 genes and the ori, E1 and ori, or E1 and multimerized E1 binding sites. A previous study showed that the presence of a strong heterologous promoter/enhancer sequence inhibited replication of BPV-1.
vectors and resulted in its integration into the host genome (7). We have recently seen a similar inhibitory effect of active promoters with HPV-1a vectors in transient replication assays. We are investigating the nature of this inhibition and attempting to overcome it using weaker promoters. However, HPV-18 vectors are clearly able to replicate in the presence of transcriptionally active regions.

So far, a system for HPV virion production using transfected DNA has not been reported, although raft culture systems have been established that allow viral DNA replication and virion production using HPV-infected primary cells or cell lines (27–29). Clearly the development of a tissue culture system for production of virions from transfected HPV DNA would greatly facilitate the use of these vectors for gene therapy. Currently, the use of physical delivery methods such as calcium phosphate precipitation, electroporation, lipofection, and bioballistics (30) would appear to provide the best means for the use of HPV vectors for gene delivery into cells.

2. Materials

1. Medium and cell lines: Human epithelial cell lines C-33A, 293, HeLa, and IB-3 are grown as monolayers in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum along with penicillin, streptomycin, and glutamine. C-33A and HeLa are human cervical carcinoma cell lines, whereas 293 is an adenovirus type 5 transformed human embryonic kidney cell line. IB-3 is an SV40 T-antigen-transformed human bronchial epithelial cell line derived from a cystic fibrosis patient.

2. 2X BBS: 50 mM BES, 280 mM NaCl, and 1.5M Na_2HPO_4, pH adjusted to 6.96 with NaOH. Filter sterilize and store at -20°C.

3. Phosphate-buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 (anhydrous), and 0.2 g K_2HPO_4 (anhydrous)/L.

4. 0.25M CaCl_2.

5. Hirt lysis solution: 0.6% SDS, 10 mM EDTA.

6. Proteinase K.

3. Methods

3.1. DNA Transfections

To test the ability of HPV-based vectors to replicate, replication analysis is carried out in appropriate cell lines using the calcium phosphate transfection method (31).

1. Plate cells at a density of 2–4 × 10^5 cells on a 60-mm plate and incubate overnight in 5% CO_2 at 37°C.

2. Mix 5 µg of the HPV vector DNA with 0.25 mL of 0.25M CaCl_2 and 0.25 mL of 2X BBS. Let stand at room temperature for 15–20 min.
3. Add the calcium phosphate-DNA solution drop-wise to the cells with constant swirling to mix well.
4. Incubate cells at 37°C overnight in 2% CO₂.
5. Remove medium and rinse cells three times with DMEM (1% serum). Feed cells with normal medium.
6. Incubate cells at 37°C in 5% CO₂ for 3–4 d for transient replication analysis. For stable transfection, start selection with appropriate concentration of the drug in the medium after 1 d. Stable colonies should be visible after approx 10 d of selection in the medium.

Replication of the HPV DNA is assayed by the isolation of Hirt fraction (32) and Southern blot analysis (33) as described in Section 3.2.

3.2. Replication Analysis

The ability of the various HPV vectors to replicate extrachromosomally in transfected cells is tested as follows:

1. Wash the cells obtained after transient or stable transfection as described in Section 3.1 with PBS, and treat with 0.5 mL of Hirt lysis solution.
2. Scrape the cell lysate into a microcentrifuge tube and add 150 μL of 5M NaCl Mix and incubate at 4°C for 2 h.
3. Centrifuge for 30 min in a microcentrifuge, collect the supernatant, and treat with 50 μg/mL of proteinase K at 37°C for 4 h.
4. Extract samples twice with phenol/chloroform/isoamylalcohol (25:24:1) and recover the DNA by alcohol precipitation.
5. To distinguish between replicated and unreplicated DNA, treat one-half of each sample with an excess of the restriction endonuclease DpnI. DNA samples can also be linearized with an enzyme to convert the various forms of HPV DNA into a single band.
6. Analyze the DNA samples by agarose gel electrophoresis and Southern blot hybridization using an appropriate probe generated by random primer labeling.

4. Notes

A number of important factors should be considered for the design of HPV vectors. For example, the direction of transcription of the E1 and E2 genes with respect to each other and the origin affects the efficiency of replication. This is evident from the observation that plasmid pFS106 replicates to much higher levels than pFS105. Also, the strength of promoters driving the E1 and E2 genes affects replication. Therefore, it is important to use different promoters for the expression of the E1 and E2 genes in HPV vectors to optimize their replication. It may be desirable to use a single transcription unit for the expression of the E1 and E2 genes. This could be accomplished by the placement of an internal ribosome entry site sequence from the encephalomycarditis virus between the E1 and E2 genes, immediately upstream of the initiating ATG.
Human Papillomavirus Vectors 123
codon of the E2 ORF. The use of various origin subregions of HPVs may also provide a means for modulating the copy number of these vectors. We are also testing the cloning capacity of the HPV vectors that are still able to replicate stably in an extrachromosomal form. It is known that BPV-1 and HPVs can attach to a wide range of mammalian cell lines of both fibroblastic and epithelial origin (34). This interaction presumably involves a conserved cell surface receptor. The development of a tissue culture system for the production of viral particles from transfected HPV DNA will clearly provide an impetus for further development of such vectors. Since no packaging system for HPVs is currently available, introduction of such vectors into cells usually involves calcium phosphate precipitation, electroporation, lipofection, and bioballistics. Other potential delivery systems would include packaging of HPV vectors into infectious adenovirus or herpesvirus particles and delivery into target cells. This would involve cloning of recombinogenic sequences surrounding the HPV DNA that would promote the removal and circularization of the vector DNA after delivery into target cells. Such efforts are currently underway in our laboratory.

Recent studies demonstrating the ability of transfected PV DNAs to replicate in a wide range of mammalian cell lines of fibroblastic and epithelial origin have generated a renewed interest in papillomavirus vectors. It may be possible to develop PV vectors that replicate in other types of cells by ensuring the expression of the E1 and E2 genes using promoters that are active in various cell types. Continued development of these vectors may lead to the exciting prospect of their use in gene therapy involving various tissues.

References


Methods for Liposome-Mediated Gene Transfer to the Arterial Wall

Elizabeth G. Nabel, Zhi-yong Yang, Hong San, Dianne P. Carr, and Gary J. Nabel

1. Introduction

Cationic liposomes are preparations of positively charged lipids used for transfection of mammalian cells in vitro and in vivo. Although viruses efficiently infect mammalian cells, the potential risks of viral gene delivery spurred research in the development of synthetic chemical vectors that would duplicate viral delivery but have no risk of infectious complications. These nonviral vectors were first developed in the late 1980s. Cationic lipids are attractive vectors for vascular gene transfer studies owing to ease of preparation, reproducibility, and safety (1–3). In this chapter, we describe our experience with vascular gene transfer using four liposome reagents: DOTMA/DOPE, DC-cholesterol, DOSPA/DOPE, and DMRIE/DOPE.

Cationic liposomes interact spontaneously and rapidly with polyanions such as DNA, mRNA, and antisense oligonucleotides to form liposome complexes. One of the first reagents developed is DOTMA (N-[1-{2,3-dioleyloxy}propyl]-N,N,N-trimethylammonium chloride)/DOPE (dioleoyl-phosphatidylethanolamine)/DOPE (dioleoyl-phosphatidylethanolamine), mixed in equal molar ratios (4) (Lipofectin, Gibco-BRL, Gaithersburg, MD). Additional cationic liposomes reagents used in vascular gene transfer studies include DC-cholesterol (dioleoyl phosphatidylethanolamine [DOPE]/3b[N-2{N,N'-dimethylaminoethane}-carbamoyl]cholesterol); DOSPA/DOPE (2,3-dioleyloxy-N-[2{ spermine carboxamido} ethyl]-N,Ndimethyl-1-propanaminiumtrifluoroacetate [DOSPA]/dioleoyl phosphatidylethanolamine [DOPE]) (Gibco-BRL); and DMRIE/DOPE (1,2dimyristyloxypropyl-3-dimethylhydroxyethyl ammonium bromide [DMRIE]/dioleoyl phosphatidylethanolamine [DOPE]) (Vical, San Diego, CA).

From Methods in Molecular Medicine, Gene Therapy Protocols
Edited by P Robbins Humana Press Inc, Totowa, NJ

127
Cationic liposomes were developed, in part, to overcome some of the deficiencies of neutral and anionic liposomes. Problems associated with the use of neutral and anionic liposomes for DNA delivery were low encapsulation efficiency and lysosomal degradation of liposomes and DNA (5). DNA encapsulated by cationic liposomes achieve a much higher concentration in target tissues than the same agent incorporated in neutral or anionic liposomes. Cationic liposomes have geometry and physical properties conducive to stable bilayer formation. The entrapment of DNA into the aqueous compartment of the cationic liposomes is not dependent on or limited by the size of the DNA. The development of this condensation technology with cationic liposomes avoids some of the encapsulation efficiency problems associated with anionic or neutral vesicles. Cationic liposomes are small in size (approx 250 nM) and homogeneous, thereby delivering polynucleotide more uniformly over target cells. An important property of cationic liposomes is fusion with cells in a manner that escapes degradation in lysosomes. DNA liposome complexes enter cells by either direct fusion with plasma membrane or by fusion with endosomal membranes after endocytosis. Following release from lysosomes, plasmid DNA is maintained in an extrachromosomal form.

Cationic liposomes have been employed in arterial gene transfer studies in vivo in several animal models including normal and atherosclerotic arteries of rats (6), rabbits (7,8), dogs (9,10), and pigs (11–15). There are several advantages of cationic liposomes for vascular gene transfer studies. An important feature of liposome vectors is the favorable safety profile. Liposome vectors contain no viral sequences. There are no cDNA size constraints in vector construction. Cationic liposomes have been administered intravenously and intra-arterially with minimal biochemical, hemodynamic, or cardiac toxicity (1,2,16). In addition, cationic liposome and DNA complexes are straightforward to prepare for experimental use.

In general, approx 5–15% of endothelial cells and smooth muscle cells are transfected in vitro with liposome vectors. The efficiency of transfection in HUVECs is lower, ~1–5% in vitro. In porcine arteries in vivo, the efficiency of gene transfer is 0.1–1%. Following liposomal transfection, gene expression is observed for approx 1 mo. Cell division is not required for liposome transfection, although the efficiency appears to be increased in proliferating cells (17). Further modifications to the chemical formulation of cationic liposomes appear promising in terms of improvement of transfection efficiencies. Complexing a heat-inactivated hemagglutinating virus of Japan (HVJ) with cationic liposomes improves transfection efficiencies in a rat arterial model of gene transfer (6).
Liposome-Mediated Gene Transfer 129

2. Materials

1. DOTMA/DOPE: N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE) (Lipofectin, Gibco-BRL, Gaithersburg, MD).

2. DC-cholesterol: dioleoyl phosphatidylethanolamine (DOPE)/3b[n(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (Dr L Huang, University of Pittsburgh, Pittsburgh, PA)

3. DMRIE/DOPE. 1,2-dimyristoylpropyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE)/dioleoyl phosphatidylethanolamine (DOPE) (Vical).

4. DOSPA/DOPE: 2,3-dioleoyloxy-N-[2(spermine carboxamido) ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA)/dioleoyl phosphatidylethanolamine (DOPE) (Lipofectamine, Gibco-BRL).

5. Opti-MEM.

6. Medium 199

7. Dulbecco’s Modified Eagle’s Medium (DMEM)


9. Other media additives: collagenase, trypsin, EDTA.

10. Dispersing medium: 2 mg/mL collagenase, 0.1 mg/mL elastase, 0.5 mg/mL soya bean trypsin inhibitor, 1 mg/mL bovine serum albumin (defatted), 20 U/mL penicillin, and 100 μg/mL streptomycin in M199.

11. Ca\(^{2+}\) and Mg\(^{2+}\)-free Dulbecco’s phosphate buffered saline (Pi/NaCl)

12. 0.2% gelatin-coated T25 flask.

13. DMSO.


15. Alkaline phosphatase substrate solution: PBS containing 5-bromo 4-chloro 3-indolylphosphate-p-toluidine (1 mg/mL) (Gibco-BRL) and nitroblue tetrazolium chloride (1 mg/mL)

16. X-gal substrate: dissolve 2 mg X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) in 0.5 mL dimethylformamide to a final concentration of 20 mg/mL. For 100 mL, 2.5 mL of X-gal solution is added to 5.0 mL 1M Tris-HCl pH 7.5, 0.2 mL of 5M NaCl, 0.1 mL of 1M MgCl\(_2\), 5.0 mL of 2.5M ferriferrocyanide, and 87.1 mL distilled H\(_2\)O.

3. Methods

3.1. Derivation and Culture of Porcine Endothelial Cells

Primary cultures of porcine endothelium are derived from the carotid artery, pulmonary artery, coronary artery, and aorta of Yorkshire pigs. The arteries are placed in HEPES-buffered M199 with gentamicin and amphotericin B and transported to the laboratory on ice. The artery is rinsed in sterile PBS (pH 7.4) until all blood is washed off. Excess tissue is removed from the artery. The
artery is placed in 10 mL serum-free M199 containing collagenase, 0.5 μg/μL, and kept at 37°C for 15 min. The artery is laid flat and opened lengthwise. The endothelium is gently scraped with a #10 scalpel blade to remove endothelial cells.

Cells removed by this means are collected by centrifugation (4°C at 800g for 10 min) and resuspended in M199 supplemented with 20% FBS, 2 mM glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, 150 ng/mL endothelial cell growth supplement and 90 μg/mL heparin. The cells are placed into a 0.2% gelatin-coated T25 flask and incubated overnight. Cells are passaged with trypsin and 0.02 mM EDTA at a passage ratio of 1:2 or 1:3. Cell monolayers are identified as endothelial cells by phase-contrast microscopy. Representative dishes of cells are further characterized by staining for factor VIII-related antigen (vWF).

Aliquots of endothelial or smooth muscle cells are stored in liquid nitrogen by resuspending approx 10⁶ cells in 0.5 mL of ice-cold fetal calf serum on ice. An equal volume of ice-cold fetal calf serum containing 10% DMSO is added, and cells are transferred to a prechilled screw-cap Corning (Corning, NY) freezing tube. These cells are transferred to a -70°C freezer for 24 h before transferring to liquid nitrogen for long-term storage.

3.2. Derivation and Culture of Porcine Smooth Muscle Cells

Primary vascular smooth muscle cell cultures are also generated from domestic Yorkshire pigs. Vascular smooth muscle cells are enzymatically derived from a 10-cm segment of artery or vein. The vessel is rinsed in M199 containing 200 U/mL penicillin and 200 μg/mL streptomycin and then incubated without agitation for 30 min at 37°C in 4 mL of dispersing medium: 2 mg/mL collagenase, 0.1 mg/mL elastase, 0.5 mg/mL soybean trypsin inhibitor, 1 mg/mL bovine serum albumin (defatted), 200 U/mL penicillin, and 100 μg/mL streptomycin in M199. The adventitia is removed with the aid of forceps as an intact, everted tube. The remaining media and intima are rinsed with M199, placed in 5 mL of fresh dispersing medium for an additional 30–60 min at 37°C, and rinsed again in M199. The medial tube is minced and incubated for 2–3 h in fresh dispersing medium. The isolated cells are washed twice and resuspended in M199 containing 10% fetal bovine serum. The procedure yields 0.5–1.5 × 10⁶ cells, which are transferred to a 75-cm² tissue culture flask. Stock cultures are passaged by washing once with 2 mL Ca²⁺ and Mg²⁺-free Dulbecco’s phosphate-buffered saline (Pi/NaCl) and incubating for 5 min at 37°C with 1 mL 0.05% trypsin in Pi/NaCl containing 0.02% Na EDTA. The cultures are passaged twice weekly and are used for experiments between the 4th and 15th passages. The stock cultures are grown in M199 containing 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.
3.3. In Vitro Transfection of Porcine Endothelial and Smooth Muscle Cells (see Note 1)

Porcine endothelial and smooth muscle cells are transfected with DNA liposome conjugates at 50–75% confluency. Plasmid DNA is transfected into endothelial and smooth muscle cells by first diluting the cationic liposome in 0.5 mL Opti-MEM (Gibco-BRL, Grand Island, NY) at room temperature. The following concentrations of cationic liposomes are used: 12 μg 1 mg/mL DOTMA/DOPE; 10 μg 1 mg/mL DC-cholesterol; 10 μg 1 mg/mL DMRIE/DOPE; and 10 μg 2 mg/mL DOSPA/DOPE. The DNA (stock concentration >1 mg/mL) is diluted into 0.5 mL Opti-MEM media, and is added to the cationic liposome solution to a final volume of 1.0 mL. The DNA liposome complexes are mixed by gentle tapping, and left at room temperature for 5–10 min. The attached cells are washed twice with OptiMEM. The cells are incubated with the DNA liposome solution at 37°C for 3–5 h. The media is removed, and the cells are maintained in M199 and 10% calf serum for 1–2 d (see Note 1).

3.4. In Vivo Transfection of Arteries (see Note 2)

Direct gene transfer in porcine iliofemoral arteries is performed using the following protocol. Domestic Yorkshire pigs are anesthetized and intubated and, using sterile surgical techniques, the iliofemoral arteries are exposed bilaterally. A double balloon catheter (USCI, Billerica, MA) is inserted through a side branch and positioned in the iliofemoral artery. The proximal balloon is positioned just below a side branch, which is an anatomical marker. There are no side branches between the double balloons that would allow for runoff of transfection solution. The proximal balloon is inflated with 1 cc of air (180 mm Hg), and the arterial segment is rinsed with 5 mL saline and 5 mL Opti-MEM to clear the vessel of blood. Approximately 10 min prior to the insertion of the catheter, the DNA liposome conjugates are prepared as described in Section 3.3. The distal balloon is inflated at 180 mm Hg, and the solution is instilled into the arterial space between the balloons at 150 mm Hg, measured by a pressure transducer, and allowed to incubate for 20 min. Following incubation, the catheter is removed, and the arterial circulation is restored. Both the left and right iliac arteries are transfected in each animal. The animals recover for 3 wk.

At the time of sacrifice, tissue samples are prepared in a variety of ways to accommodate the requirements of the specific assay: PCR, in situ hybridization, immunohistochemistry, morphometry, light microscopy, and scanning/transmission EM. Tissue samples for DNA and RNA isolation were snap frozen in liquid nitrogen and stored at −70°C until used. Systemic organs are
biopsied for analysis by light microscopy. Separate sterile, clean instruments and gloves are used to biopsy each organ in order to avoid contamination of nontransfected tissues. In addition, samples of blood are obtained prior to gene transfer and at the time of sacrifice for biochemical analyses including glucose, electrolytes, and renal and hepatic function.

3.5. Histochemical Detection of Alkaline Phosphatase Activity

We have used two reporter genes in our vascular gene transfer studies: a human placental alkaline phosphatase gene and a nuclear targeted lacZ gene.

Human placental alkaline phosphatase is heat stable at temperatures above 65°C and can be differentiated from endogenous forms of alkaline phosphatase protein by heat inactivation. Histochemical staining in transfected cells is performed 2 d following transfection with a human placental alkaline phosphatase gene. Cells are washed once in PBS and lifted off the plate using 2 mM EDTA + PBS. Cells are removed from the culture dish, spun down, and fixed in 10% buffered formalin. The fixed cells are washed once with PBS and divided to form two cell pellets. One pellet is embedded in paraffin, and the other is embedded in optimal cutting tissue processing medium (O.C.T., Miles Diagnostic, Elkhart, IN) and stored at -80°C. Tissue specimens from harvested organs are fixed in 10% buffered formalin for 16 h, placed in 70% ethyl alcohol and embedded in paraffin. Paraffin blocks are sectioned at 6 μm thickness onto poly-L-lysine slides. The slides were deparaffinized in three changes of xylene, rehydrated in 100, 95, and 75% ethyl alcohol, and incubated in PBS at 65°C for 60 min to inactivate endogenous alkaline phosphatase. The sections are incubated in substrate solution for 19 h. This substrate yields a dark blue-purple stain. Sections are rinsed with PBS and counterstained with a neutral red stain.

3.6. Histochemical Detection of β-Galactosidase Enzyme Activity

Endothelial or smooth muscle cells are fixed in 1.25% glutaraldehyde in PBS for 5 min. Tissues are fixed in 1.25% glutaraldehyde and 4% paraformaldehyde for 20 min. Cells and tissues are rinsed in PBS three times and are incubated in X-gal substrate at 37°C for 2–6 h. Cells may be stored at 4°C in phosphate-buffered saline, or counter-stained with neutral red. Tissues are embedded in paraffin, sectioned at 6 μm thickness, and counterstained with neutral red. Alternatively, tissues are frozen in O.C.T. embedding compound, sectioned at 10–12 μm thickness, fixed in 1.25% glutaraldehyde, and stained with X-gal solution.
4. Notes

1. The ratio of cationic liposomes to DNA is an important determinant of transfection efficiency. Titration of DNA to liposome concentration should be performed with each cationic liposome preparation and each cell line in vitro. Differences in target cell, plasmid, and cationic lipid will require adjustment to the DNA lipid ratio. In general, we optimize the liposome DNA ratio in vitro on the desired target cell, and use this optimal ratio for in vivo studies.

2. When performing in vivo arterial gene transfer, having serum-free conditions at the site of transfection is important. When using a double catheter, we flush the inner space between the two balloons with Opti-MEM to rid the space of serum components. Cationic liposomes can be inactivated by serum proteins. If it is technically impossible to rid the transfection site of serum, rinsing the tissue with Opti-MEM is preferable.

References

Methods for Targeted Gene Transfer to Liver Using DNA-Protein Complexes

Mark A. Findeis, Catherine H. Wu, and George Y. Wu

1. Introduction

The potential of therapeutic gene transfer to treat human disease has prompted a diverse and growing range of basic and applied research efforts to explore and develop gene therapy strategies (1-5). Reported approaches to gene therapy include the uses of retroviruses (6,7), adenovirus (8,9), receptor-mediated endocytosis (10,11), direct injection (12), and liposomes (13,14), among others. Targeted delivery of DNA via receptors has been successfully applied using protein ligands to the hepatic asialoglycoprotein receptor (ASGr) (10,15-18), and, subsequently, the transferrin receptor (11). The ASGr is a cell-surface receptor that is highly represented on hepatocytes. Thus, genes targeted to this receptor can be delivered in a highly selective manner to the liver.

The practicality of receptor-mediated targeted delivery via the hepatic ASGr was demonstrated over a decade ago for the delivery of agents covalently bound to carriers such as asialofetuin (19-21) and asialoorosomucoid (22) or galactose-terminated neoglycoproteins (23,24). This strategy was then extended to the use of asialoorosomucoid-polylysine (ASOR-PL) conjugates to carry DNA into hepatocytes (15-18). Targeted delivery by this technique has been used successfully in vitro (10,25-27) and in vivo (28-32) for the delivery of DNAs coding for a variety of genes.

In a refinement of receptor-mediated gene delivery, we have used adenovirus as an additional component of ASGr-targeted DNA complexes to achieve enhanced gene delivery and expression (33). Adenoviruses are common pathogens in man and animals (34,35) and have broad tissue specificity (36). The
virus normally enters cells by binding to plasma membrane of the host cell through adenoviral fibers that project from the capsid (37). Binding triggers internalization of viral particles within endosomal vesicles (38). Acidification of endosomes results in a conformational change of the capsid proteins that disrupts the endosomal membrane (39). This event permits the viral nucleic acid to enter the cytosol of the host cell prior to exposure to lysosomal enzymes that would occur normally during endocytosis. The endosomolytic property of adenoviruses has been used previously to enhance the delivery of polypeptides (39) and more recently plasmid DNA (40). However, in the latter instance, the DNA complex retained the original infection specificity of the virus in addition to that of the ligand.

Because the viral fibers govern the infection specificity of adenovirus, chemical coupling of an asialoglycoprotein to these structures was expected to result in a new ability to enter cells by the ASGr while simultaneously blocking recognition of the cells' endogenous receptors for the virus. Our strategy (Fig. 1) was to take advantage of the fact that the adenovirus contains carbohydrate groups that are exclusively located on the fibers (41). Mild oxidation of the carbohydrate residues allows covalent linkage of an asialoglycoprotein-polylysine conjugate to the surface of the virus. Addition of foreign DNA results in electrostatic binding to the polylysine of the conjugate and coating of the modified virus by the DNA to form a targetable complex. Internalization of the complex is achieved in a cell-specific fashion mediated by receptor recognition of the asialoglycoprotein component of the complex and enhanced by the endosomolytic properties of the virus.

ASOR is readily available by isolation of orosomucoid (also referred to as α1-acid glycoprotein) from human plasma (42) followed by desialylation to expose penultimate galactose groups. Although orosomucoid is commercially available, it is readily isolable from plasma in good yield (42). Orosomucoid can be desialylated by a simple acid treatment with heating, thus avoiding the expense of using neuraminidase to cleave sialic acid residues (43). Conjugates formed by crosslinking polylysine (PL) with proteins have been reported using carbodiimide-mediated amide bond formation (26,27) and thiol reagents (10,11,25). The product mixtures obtained in the formation of conjugates of this type are heterogeneous and highly charged. Previously described methods for the preparation of ASOR-PL targeted to the ASGr have used dialysis (22), gel-filtration (22,25), and ion-exchange HPLC (11,26) for purification of the conjugate from reaction byproducts and residual starting materials. We describe here techniques for the preparation of ASOR-PL conjugates and of adenovirus-ASOR-PL conjugates that effectively bind DNA in an electrostatic complex for targeted delivery of DNA to ASGr-bearing cells (44).
DNA-Protein Complexes in Gene Transfer

Fig. 1. Formation of adenovirus-ASOR-PL-DNA complexes. Step 1: Mild oxidation is used to modify carbohydrate on viral fibers. Step 2: ASOR-PL conjugate is chemically coupled to the modified carbohydrate. Step 3: Adenovirus-ASOR-PL conjugate is combined with DNA to form a complex targeted to the asialoglycoprotein receptor. Reprinted with permission from ref. 33.

2. Materials

2.1. Isolation of Orosomucoid

1. Pooled human plasma (American Red Cross, Farmington, CT)
2. DEAE and CM Sepharose beads (Millipore, Milford, CT).
3. Spectra/Por2 dialysis tubing (12–14 kDa mol-wt cut-off) (Spectrum Industries, Los Angeles, CA)

2.2. Chemical Coupling of Asialooromucoid to Polylysine

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC) (Sigma, St. Louis, MO).

2.3. Adenovirus

Type 5 adenovirus (from Dr. Hamish Young, Columbia University, New York).

2.4. Cell Lines

1. HeLa S3 cells (American Type Culture Collection/NIH, Rockville, MD).
2. SK Hep1 cells (from Dr. David Shafritz, Albert Einstein College of Medicine, Bronx, NY).
3. Huh7 cells (from Dr. T. Jake Liang, Massachusetts General Hospital, Boston, MA).

2.5. Assay for Hepatitis B Surface Antigen

Hepatitis B surface antigen ELISA kits (Abbott Laboratories, North Chicago, IL).

2.6. β-Galactosidase Marker Gene

1. Plasmid βact nls lacZ A1 (from Dr. Claire Bonnerot, Pasteur Institute, Paris, France).
2. X-gal (Life Technologies, Gaithersburg, MD).

3. Methods

3.1. Preparation of Human Orosomucoid (α1-Acid Glycoprotein)

Buffers used in this isolation contain the stated concentration of sodium acetate (NaOAc) and added glacial acetic acid to obtain the desired pH: Buffer 1, 0.05M
NaOAc, pH 4.5; Buffer 2, 0.10M NaOAc, 0.05M NaCl, pH 4.0; Buffer 3, 0.1M NaOAc, 1.0M NaCl, pH 4.0. These buffers are slightly different from those used with DEAE cellulose anion-exchange media (42). Buffer 2 (0.10M NaOAc, pH 4.0) without 0.05M NaCl added was used as an additional wash step prior to the elution of orosomucoid (OR). The OR-rich fraction was eluted with 0.10M NaOAc, 0.10M NaCl, pH 4.0, then purified by ammonium sulfate precipitation.

DEAE Sepharose Fast Flow and CM Sepharose Fast Flow media were packed in 5-cm diameter by 30-cm height Waters AP-5 columns (Millipore) according to the manufacturer's instructions. The DEAE Sepharose column was equilibrated with Buffer 1, and the CM sepharose column was equilibrated with Buffer 2. Buffer was pumped through the column with a Rainin Rabbit-Plus peristaltic pump (Rainin, Woburn, MA) equipped with a high-flow pump head fitted with 5.0-mm diameter silicone tubing at a flow rate of 45 mL/min.

Pooled human plasma (4 U, ~1.2 L, outdated human plasma from the American Red Cross) was transferred to Spectra/Por 2 dialysis tubing (12–14 kDa mol wt cutoff from Spectrum) and dialyzed overnight at 4°C against 20 L of Buffer 1. The dialyzed plasma was then centrifuged in a Beckman JA-14 rotor (Beckman Instruments, Fullerton, CA) at 30,100g for 15 min at 4°C. The supernatant was then filtered through Whatman #2 paper (Whatman, Clifton, NJ), followed by an additional filtration through a 0.45-μm nylon Zapcap filter (Schleicher & Schuell, Keene, NH). The precipitate was discarded. The dialyzed and filtered plasma was applied to the DEAE sepharose column at a flow rate of 25 mL/min (a lower flow rate is required owing to the high viscosity of plasma). The column was then washed with Buffer 1 until the eluate had an absorbance at 280 nm of <0.10 cm⁻¹. The column was then washed with Buffer 2. The OR-rich eluate was collected starting when the A₂₈₀ began to increase and ending after the A₂₈₀ had peaked and was <0.10 cm⁻¹.

The OR-rich Buffer 2 eluate was then applied to the CM sepharose column (previously equilibrated with Buffer 2), which was then washed with Buffer 2. The eluate was collected starting when the A₂₈₀ began to increase and ending after the A₂₈₀ had peaked and was <0.10 cm⁻¹. After this purified OR fraction was collected, the CM Sepharose column was washed with Buffer 3 (1 L).

The purified OR was concentrated tenfold and then diafiltered with 10 vol of water in an Amicon CH2 Hollow Fiber Concentrator fitted with an Amicon S1Y10 Spiral Membrane Ultrafiltration Cartridge (Amicon, Beverly, MA). The desalted OR fraction was then lyophilized and stored at -20°C. A sample of the OR was run on SDS-PAGE (45,46) and showed a single band at the expected apparent mol wt of 44 kDa by staining with Coomassie blue. The actual mol wt
of OR has been reported in the range of 41,000 kDa (47,48), and more recently was determined by mass spectrometry to be 36,800 (49). Although there is some variability in the amount of OR in human plasma samples, the typical yield of lyophilized salt-free OR using this procedure is ~600 mg.

### 3.2. Preparation of Asialoorosomucoid

Asialoorosomucoid (ASOR) is prepared by cleavage of terminal sialic acid residues from the branched carbohydrates on the surface of OR (43). The OR (10 mg/mL) isolated as above is dissolved in water. An equal volume of 0.1N sulfuric acid (2.78 mL/L of concentrated H$_2$SO$_4$ in water) is added to the OR solution, and the resulting mixture is heated at 80°C for 1 h in a water bath to hydrolyze sialic acids from the protein. The acidolysis mixture is removed from the water bath, neutralized with NaOH solution, dialyzed against water for 2 d, and then lyophilized. The thiobarbituric acid assay of Warren (50), or Uchida (51), is then used to verify desialylation of the OR. Targetability of ASOR samples is verified by labeling with $^{125}$I and measuring liver uptake in rats or mice (28).

### 3.3. Synthesis of Asialoorosomucoid-Polylysine Conjugates

#### 3.3.1. Method A

The ASOR (166 mg) is dissolved in water (5 mL), and the solution is filtered through a 0.45-μm syringe filter. The filter is washed with water (5 mL), the filtrates combined, and the pH is adjusted to 7.4 with 0.1N NaOH. Poly-L-lysine (PL, as the HBr salt, 37 mg) of average 25 kDa mol wt is dissolved in water (5 mL), and the pH of the solution is adjusted to 7.4 (see Note 1). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC, 25 mg) is dissolved in water (0.33 mL) and added directly to the ASOR solution. The PL solution is added to the ASOR-EDC solution and the pH is readjusted to 7.4. Additional EDC (25 mg in 0.33 mL of water) is added after 2 h and 3 h. The reaction mixture is covered and maintained at 37°C for 72 h total. The reaction mixture is then transferred to 12–14 kDa mol wt cut dialysis tubing and dialyzed at 4°C against water (20 L) for 2 d with one change of water. The dialysate is lyophilized (169 mg) prior to further purification (Method A).

#### 3.3.2. Method B

The ASOR and PL (mol wt = 41,000) in a 1:1 weight ratio in 5 mL of deionized water at pH 7.4 were coupled by the addition of EDC in a 140-fold molar excess over ASOR and stirred for 16 h at 25°C. The reaction mixture was then dialyzed against deionized water at 4°C for 72 h, lyophilized, and purified (Method B).
Table 1
Recipe for the Preparation of Analytical Gels

Solutions to prepare for use in casting acid-urea gels

<table>
<thead>
<tr>
<th>Solution Description</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 M Urea</td>
<td>48 g plus H₂O to 100 mL</td>
</tr>
<tr>
<td>Running gel buffer (RGB)</td>
<td>48 mL 1M potassium hydroxide 17.2 mL glacial acetic acid water to 100 mL</td>
</tr>
<tr>
<td>4X Sample buffer (4X SB)</td>
<td>1.34 g potassium hydroxide 2 mL glacial acetic acid water to 100 mL</td>
</tr>
<tr>
<td>10X Running buffer (10X RB)</td>
<td>311.8 g β-alanine 80.4 mL acetic acid water to 1 L</td>
</tr>
<tr>
<td>30% Bis/acrylamide</td>
<td>30 g acrylamide 0.8 g bisacrylamide 100 mL H₂O</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>100 mg in 1 mL H₂O</td>
</tr>
<tr>
<td>Methyl green stock</td>
<td>0.1% methyl green in 1:3 (4X SB in 8M urea)</td>
</tr>
<tr>
<td>Coomassie stain</td>
<td>1 g/L in 40% ethanol, 10% acetic acid in H₂O</td>
</tr>
<tr>
<td>Destaining solution</td>
<td>10% ethanol, 7.5% acetic acid in H₂O</td>
</tr>
</tbody>
</table>

Gel recipe (for 10% running gel, 4% stacking gel)

<table>
<thead>
<tr>
<th>Solution Description</th>
<th>Running Gel</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Bis/acrylamide</td>
<td>13.3 mL</td>
<td>1.3 mL</td>
</tr>
<tr>
<td>Buffer</td>
<td>5 mL RGB</td>
<td>2.5 mL 4X SB</td>
</tr>
<tr>
<td>8 M urea</td>
<td>20 mL</td>
<td>5 mL</td>
</tr>
<tr>
<td>H₂O</td>
<td>1.2 mL</td>
<td>1.3 mL</td>
</tr>
<tr>
<td>10% Ammonium persulfate</td>
<td>0.3 mL</td>
<td>0.1 mL</td>
</tr>
<tr>
<td>TEMED&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2 mL</td>
<td>0.012 mL</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ref 46, p 68
<sup>b</sup>Proportions of solutions in (A) to use for casting running gels and stacking gels, respectively
<sup>c</sup>N,N,N',N'-Tetramethylethylenediamine.

3.4. Analytical Acid-Urea Gel Electrophoresis

Analytical gels are prepared using the recipe in Table 1 adapted from the method of Panyim and Chalkley (52). Prior to the addition of TEMED, acrylamide solutions are degassed under vacuum (~20 mmHg) for 10 min. After samples are loaded on cast gels, electrophoresis is run from anode to cathode (electrodes connected in reverse polarity from the usual). Voltage is at 90–150 V. Methyl green can be used as a tracking dye in these gels, although we usually omit it. Gels are stained with 0.1% Coomassie brilliant blue R250 in ethanol-acetic acid-water (40:10:50 v/v).
DNA-Protein Complexes in Gene Transfer

Fig. 2. Elution profile from preparative acid-urea elution electrophoresis. The sample was a crude product mixture (169 mg) from the cross-linking of ASOR with 25 kDa average mol wt PL as described in Method A. Fractions (10 mL) were collected starting between peaks 1 (the ion front and unbound PL) and 2. Peaks 2 (fractions 5–17, 27 mg) and 3 (fractions 19–43, 43 mg) were pooled, dialyzed against water, and lyophilized before further fractionation by cation-exchange HPLC. Reprinted with permission of the American Chemical Society (44).

3.5. Purification of ASOR-PL Conjugates

3.5.1. Method A

3.5.1.1. Preparative Acid-Urea Gel Elution Electrophoresis

Preparative gels are cast and run in the Bio-Rad (Hercules, CA) Model 491 Prep Cell apparatus (recipe, Table 1) in a manner similar to that used for analytical gels and according to the instructions for the use of the apparatus. Running gel solutions are degassed under vacuum, poured into the gel holder, and allowed to stand overnight to ensure full polymerization. Stacking gel solutions are degassed with nitrogen gas bubbling prior to the addition of TEMED and allowed to polymerize for 30 min. After assembly of the elution electrophoresis apparatus (with the electrodes connected in reverse polarity), the elution chamber is eluted with a peristaltic pump at 1 mL/min, the sample is loaded, and electrophoresis is conducted at 11 W for small gels and 22 W for large gels. The eluate is passed through a UV detector operating at 280 nm. Fractions (5 mL) are collected starting after the ion front (first sharp peak) is eluted from the gel (Fig. 2) (44).
### Table 2

**Conditions for HPLC Purification of ASOR-PL Conjugates**

<table>
<thead>
<tr>
<th>Time, min</th>
<th>%A</th>
<th>%B</th>
<th>%C</th>
<th>%D</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25</td>
<td>0</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>25</td>
<td>0</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>25</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>25</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>0</td>
<td>25</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>35</td>
<td>0</td>
<td>25</td>
<td>70</td>
<td>5</td>
</tr>
<tr>
<td>36</td>
<td>0</td>
<td>25</td>
<td>65</td>
<td>10</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>25</td>
<td>65</td>
<td>10</td>
</tr>
<tr>
<td>51</td>
<td>0</td>
<td>25</td>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td>65</td>
<td>0</td>
<td>25</td>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td>66</td>
<td>0</td>
<td>25</td>
<td>55</td>
<td>20</td>
</tr>
<tr>
<td>80</td>
<td>0</td>
<td>25</td>
<td>55</td>
<td>20</td>
</tr>
<tr>
<td>81</td>
<td>0</td>
<td>25</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>95</td>
<td>0</td>
<td>25</td>
<td>25</td>
<td>50</td>
</tr>
</tbody>
</table>

*For cation-exchange chromatography on Waters CM 15HR packing in a 10-mm diameter × 100-mm length column. Flow rate = 1.8 mL/min. Buffers: (A) 0.4M sodium acetate, pH 5.0, (B) 0.4M sodium acetate, pH 2.3, (C) water, (D) 2M NaCl. Buffers are made up to the stated concentration in sodium acetate, pH is adjusted by adding HCl.*

#### 3.5.1.2. CATION-EXCHANGE HPLC FRACTIONATION OF ASOR-PL

Cation-exchange HPLC is performed using a 1 × 10 cm Waters AP-1 column packed with CM 15HR packing material. The gradient described in Table 2 is used to chromatograph ASOR-PL mixtures that had been freed of unreacted PL by preparative electrophoresis. Fractions of interest elute after the buffer pH is lowered to 2.3 and the salt concentration begins to increase. Typically, the first major peak observed under these conditions will have the best properties for DNA binding resulting in ASOR-PL-DNA complexes with increased solubility (Fig. 3A) (44). Figure 3B shows an analytic acid-urea gel comparing crude prep gel peak 2, in lane 2, with HPLC peaks 1–3, in lanes 2–4, respectively. Lanes 5–8 show the contents of prep gel peak 3.

**Fig. 3.** (Opposite page) (A) High-performance liquid chromatography ion-exchange chromatogram of ASOR-PL (peak 2 from Fig. 2) and associated analytical acid-urea electrophoresis gel (see Table 2 for HPLC gradient conditions). (B) The acid urea gel shows that after both preparative electrophoresis and HPLC cation-exchange, the
DNA-Protein Complexes in Gene Transfer

(Continued) fractions isolated are free of unbound polylysine (see lane A). Lane 1 corresponds to the crude sample applied to the HPLC column (peak 2, Fig. 2) and lanes 2, 3, and 4 correspond to HPLC peaks 1, 2, and 3 in Fig. 3A. The ASOR-PL isolated from peak 1 (lane 2) produces DNA complexes with greater solubility than the ASOR-PL isolated from peaks 2 and 3. Lanes 5–8 are the corresponding fractions to prep gel fraction 3 (see Fig. 2). Whereas the fraction in lane 6 elutes at a similar position to the fraction in lane 2 (chromatogram not shown), it forms DNA complexes with lower solubility. Reprinted with permission of the American Chemical Society (44).
3.5.2. Method B

3.5.2.1. Cation-Exchange HPLC Fractionation of ASOR-PL

The purification sequence was reversed with equivalent results. Chromatography was performed using an Aquapore CX-300 1.0 x 25 cm cation exchange column (Rainin) with step-wise elution at a flow rate of 4.0 mL/min with 0.1M sodium acetate, pH 5.0, 12 min; pH 2.5, 24 min; pH 2.25, 12 min; and pH 2.0, 14 min. The second peak eluted from the column, as detected by UV absorption at 230 nm, was collected. This fraction was further purified by preparative electrophoresis essentially as described in Method A. The purified conjugate was dialyzed against 0.025M Tris-0.001M EDTA (T-E) buffer through membranes with 12-14 kDa exclusion limits (44) (see Note 2).

3.6. Propagation and Preparation of Adenovirus

Type 5 adenovirus, kindly provided by Dr. Hamish Young, Columbia University, New York, was propagated and amplified in HeLa S3 cells as described previously (53). To avoid potential cytotoxic effects of wild-type virus in studies on targeted gene expression, replication defective d1312 adenoviruses were also studied after propagation in 293 cells as described previously (54).

3.7. Coupling of Adenovirus to ASOR-PL Conjugates (33)

Adenovirus samples, 1.0 x 10^{12} particles, each in a total volume of 100 μL of 0.01M Tris, 135 mM NaCl, 5 mM KCl, 1 mM MgCl2, were reacted with 100 μL of 0.02M NaIO4 for 30 min at 25°C in the dark. Then, 100 μL of 0.20M NaAsO2 in T-E buffer were added for an additional 60 min. To quantitate the amount of conjugate coupled to adenovirus, ASOR-PL conjugate was radiolabeled with Na^{125}I by a chloramine-T method (55) to a specific activity of 215 cpm/ng protein. Increasing amounts of [^{125}I]-ASOR-PL from 20 μg to 1 mg (in terms of ASOR content) in 140 μL of the adenoviral sample buffer and 100 μL of 0.02M NaBH4CN were added to adenoviral samples and incubated for 15 h at 4°C. Samples were then applied on a discontinuous CsCl gradient (56) (2.5 mL of CsCl at densities of 1.4, 1.3, and 1.2 g/mL). Samples were ultracentrifuged at 100,000g for 4 h at 4°C. Fractions (350 mL each) were collected beginning at the top of the tubes, the radioactivity determined in each sample, and the fractions dialyzed against 150 mM NaCl, 10 mM EDTA, 10 mM Tris, pH 8.0. The radioactive peak found at the interface between 1.4 and 1.3 g/mL, where the virus usually bands (56), was recovered and recentrifuged through another CsCl gradient to further purify the modified virus. Unbound conjugate remained at the top of the gradient tubes. For infectivity and gene transfection studies, virus was modified in an identical procedure using unlabeled virus in the coupling reaction.
The number of modified viral particles in each fraction was determined by quantitation of DNA by UV absorption \((57)\) after proteinase K-phenol-chloroform extraction \((58)\). In brief, purified modified virus was treated with SDS and proteinase K to make final concentrations of 0.1% and 100 \(\mu g/mL\), respectively. The samples were incubated at 37°C for 1 h followed by phenol-chloroform extraction, chloroform extraction, and precipitation with 2.5 vol of ethanol at \(-20°C\) overnight. The nucleic acid was collected by centrifugation at 8000g for 20 min at 4°C and the DNA concentration determined by measurement of the UV absorption at 260 nm. The maximum amount of ASOR-PL bound to virus was obtained with starting ratios of 20 \(\mu g\) of conjugate to \(1 \times 10^{11}\) viral particles. At this ratio, it was calculated that approx 24 molecules of conjugate were bound to each viral particle. Total protein content of the modified virus was determined by the Bio-Rad assay as instructed by the manufacturer. Purified modified virus was filtered through 0.45-\(\mu m\) membranes (Millipore) and remained stable in T-E buffer at 4°C for at least 2 wk without loss of activity.

3.8. Gel Retardation Assay (10)

Concentrations of DNA are based on an extinction coefficient of 20 \((mg/mL)^{-1}\) cm\(^{-1}\) at 260 nm, and agarose gel electrophoresis of DNA is performed as described by Sambrook \((45)\). A series of samples is prepared by adding 20-\(\mu L\) aliquots of ASOR-PL solutions in 0.15M NaCl to 20-\(\mu L\) samples of plasmid DNA at 200 ng/\(\mu L\) and 0.15M NaCl to give ASOR-PL:DNA mass ratios of 4.0:1, 3.5:1, 3.0:1, 2.5:1, 2.0:1, 1.5:1, and 1.0:1. After a 15-min incubation at room temperature, the test samples and a sample of DNA alone are electrophoresed on a 1% agarose gel eluted with 1X TPE \((45)\) at 50 V for 45 min. The optimum ASOR-PL:DNA ratio for complex formation is determined from the migration of the samples as the lowest ratio that gave 80–90% complete DNA retardation (Fig. 4A).

3.9. DNA-Conjugate Complex Formation

To form targetable complexes, two methods may be used:

1. Plasmid DNA, 0.5 mg in 1 mL of 2M NaCl, is added to ASOR-PL conjugate, 0.15 mg (with respect to ASOR content), in 600 \(\mu L\) of 2M NaCl at 25°C. The mixture is then placed in 1.0-cm (flat width) dialysis tubing with an exclusion limit of 12–14 kDa, and dialyzed step-wise successively at 4°C for 0.5 h against 1.0 L of NaCl in each of the following concentrations: 1.5M, 1.0M, 0.5M, 0.25M, and 0.15M. If the size of the plasmid is larger than 8 kb, it is advisable to dialyze more slowly, taking 24 h for each change. After the final dialysis, the complex is filtered through 0.45-\(\mu m\) membranes. Complete complexation is determined by testing samples for retardation by agarose gel electrophoresis. No free DNA should be present.
2. An ASOR-PL-DNA complex is formed at a 2:1 ASOR/PL:DNA ratio by adding 2.5 mL of an ASOR-PL solution at 1.5 mg/mL and 0.15M NaCl to a 2.5-mL stirred solution of DNA at 1.0 mg/mL and 0.15M NaCl at 25 μL/min via a peristaltic pump. After complete addition, the complex is filtered through a 0.45-μm filter and the concentration of soluble, complexed DNA is measured by UV absorbance at 260 nm (final concentration of complexed DNA: 374 μg/mL, 75% recovery). Complexation of the DNA is then confirmed by agarose gel electrophoresis (Fig. 4B). Complexes in 0.15M NaCl are usually stable at 4°C for at least 2–3 d.

3.10. Formation of Complexed DNA and Modified Virus (33)

To form targetable complexes, plasmid DNA, 0.5 mg in 1 mL of 2M NaCl was added in a ratio of 2 μg DNA to 0.4 μg modified virus, with respect to total protein. This ratio was determined as optimal using an agarose gel retardation assay as described previously (25). The sample was then placed in dialysis tubing with an exclusion limit of 12–14 kDa (Spectrapore), and dialyzed stepwise successively at 4°C for 0.5 h against 1 L of NaCl solution in each of the following concentrations: 1.5M, 1.0M, 0.5M, 0.25M, and 0.15M. After the final dialysis, the complex was filtered through 0.45-μm membranes prior to use in subsequent studies.

3.11. Cells and Cell Culture

To assess viral infectivity, three cell lines were used: human cervical carcinoma, HeLa S3 [asialglycoprotein receptor (−)], human hepatoma, SK Hep1.
[asialoglycoprotein receptor (-)] cells, and human hepatoma Huh 7 [asialoglycoprotein receptor (+)] cells (59) were cultured in plastic dishes. Cells were seeded at densities of 5 x 10^5 cells in 35-mm plastic dishes containing minimal essential medium (MEM) (Gibco-BRL, Gaithersburg, MD) and 5% fetal calf serum (Gibco-BRL) under 5% CO_2 at 37°C.

3.12. Assays of Viral Infectivity

To assess residual infectivity of modified viruses, cells were allowed to attach for 24 h, after which time medium was removed and virus, modified or unmodified in medium, was added at concentrations of 100 viral particles/cell. Virus samples were incubated with cells at 37°C for 48 h. Cells were then stained with Trypan blue and counted microscopically. Infection assays were performed in triplicate and the results expressed as mean ± SD of viable cells.

3.13. Assays for Targeted Gene Expression (33)

To assay for gene transfection, a plasmid, pHBVsurf, containing the gene for human hepatitis B surface antigen driven by the SV-40 promoter (kindly provided by Dr. T. Jake Liang, Massachusetts General Hospital, Boston, MA) was used (59). The plasmid was grown in E. coli, isolated and purified as described previously (60). Cells, seeded at 5.0 x 10^5/35-mm dish, were treated 24 h later with 2 mL of medium containing 2 μg DNA in the form of DNA complex alone, DNA complexed to modified wild-type virus, DNA complex plus modified dl312 virus, DNA complexed to modified dl312 virus, and DNA complexed to modified dl312 virus plus 1000-fold excess of ASOR by weight with respect to the ASOR content of the complex. In samples containing virus, approx 2000 viruses/cell were present and in competition studies, a 1000-fold excess of viral particles/cell was used. All transfections were performed in triplicate and parallel dishes were prepared for cell counting. Medium, 200 μL from each dish, was removed at 24 h and assayed for HBV surface antigen concentration by ELISA (Abbott, North Chicago, IL) and expressed as pg/10^6 cells/24 h ± SD.

To determine the number of cells that express targeted genes, a plasmid pTZ βact nls lacZ A1 (a kind gift of Dr. Claire Bonnerot, Pasteur Institute, Paris, France) (61) containing the gene for β-galactosidase driven by a retroviral promoter with an SV-40 nuclear localizing sequence was employed. Asialoglycoprotein receptor (+) and (-) cells, all seeded at 5.0 x 10^5 cells/dish, were transfected separately, 24 h later, with 2 μg DNA in 2 mL of medium as DNA complex alone, DNA complexed to modified dl312 adenovirus at 2000 particles/cell, DNA complexed to modified dl312 adenovirus plus a 1000-fold excess of ASOR. After 24 h of incubation, cells were fixed and washed (62), and then stained with X-gal (63) (Life Technologies, Gaithersburg, MD); the number of cells with blue-stained nuclei was determined by counting micro-
scopically. The fraction of positive cells was calculated and expressed as mean ± SD of 10 high power fields.

4. Notes

1. The procedures described here represent a general guide that accommodates considerable flexibility. In our laboratories, we have prepared conjugates using PLs over a wide range of average molecular weights (4–60 kDa) with some variation in DNA binding and delivery.

2. Care must be taken to properly purify ASOR-PL to allow the formation of a soluble DNA complex. For applications in vivo, in particular, the ASOR-PL should be free of both unbound PL and the highly cross-linked ASOR-PL species that migrate more slowly in the acid-urea gels. ASOR-PL purified in this manner allows the formation of DNA complexes at a concentration suitable for iv injection into experimental animals (up to 1 mg/mL DNA). For in vitro experimentation, for which complexes can be made up at lower concentrations, it is possible (though not preferable) to use less rigorously purified ASOR-PL to prepare soluble targetable complexes. Complexes of oligonucleotides (26, 64) are more soluble than those of plasmid DNAs and also may be prepared with less highly purified ASOR-PL.

Acknowledgments

Supported in part by grants from NIH: DK42182 (George Y. Wu), March of Dimes 1-0786 (George Y. Wu); and TargeTech (Catherine H. Wu). George Y. Wu, and Catherine H. Wu hold equity in Immune Response Corp. The technical assistance of Pei-Li Zhan is gratefully acknowledged. The data presented in Figs. 2–4 were collected by Timothy D. McKee and Mary E. DeRome (TargeTech, Meriden, CT). The purification of orosomucoid described was developed by Todd Mockler, Chris Coffin, Timothy McKee, and Mary E. DeRome (TargeTech).

References

8. Rosenfeld, M. A., Yoshimura, K., Trapnell, B. C., Yoneyama, K., Rosenthal, E. R., Dalemans, W., Fukayama, M., Bargon, J., Stier, L. E., Stratford-Perricaudet,
DNA-Protein Complexes in Gene Transfer


hepatitis virus viremia at lower doses than do the unconjugated drugs *Hepatology* 14, 16–24.


Methods for the Use of Retroviral Vectors for Transfer of the CFTR Gene to Airway Epithelium

John C. Olsen, Larry G. Johnson, and James R. Yankaskas

1. Introduction

Cystic fibrosis (CF) is a recessive genetic disease that affects the regulation of ion transport in the epithelia of various organs in the body including the lungs, pancreas, intestine, salivary glands, and urogenital tract. The protein encoded by the CF gene is an integral plasma membrane protein called the cystic fibrosis transmembrane conductance regulator (CFTR) and has been shown to function as a chloride channel (1). In the lungs, CFTR dysfunction affects electrolyte and fluid transport across the apical membrane of airway epithelial cells. There, sodium hyperabsorption and defective chloride secretion lead to dehydration of the fluids on the airway surface and, in turn, this leads to chronic infections and severe damage. The severity of CF lung disease and the potential accessibility of the airways to gene transfer vectors has led to proposals that gene therapy be applied for the treatment of CF lung disease (2).

Heterozygotes with mutations in the CFTR gene show no phenotypic abnormalities. Thus, gene transfer of a normal CFTR cDNA to CF airway epithelial cells grown in culture corrects the chloride transport defect. Although current gene therapy trials use adenoviral vectors and liposomes to deliver CFTR cDNA to CF patients in vivo, it is possible that retroviral vectors may be used in the future. Potential advantages of using retroviruses include stable integration, which could lead to long-term therapy and the unlikelihood of vector rescue by other viruses that commonly infect the respiratory tract. Disadvantages of using retroviruses include the relatively low titers that are produced by commonly used packaging cell lines and the fact that retroviruses require dividing cells for integration and the proliferation rate of epithelial cells lining the airway of normal individuals is low. However, a recent study showing that the
proliferation rate of the airway epithelium of CF patients may be quite high suggests that retrovirus vectors might indeed be useful in transferring genes directly to injured lungs (3). Alternatively, retroviral vectors may prove useful for transferring genes to fetal lungs in which the cellular proliferation rate is known to be relatively high.

In this chapter, procedures to effect and evaluate retroviral gene transfer into human airway epithelial cells are described in three sections. First, procedures are described for the preparation of primary airway epithelial cell cultures that can then be used to evaluate the gene transfer capabilities of various gene transfer vectors. Second, methods used to increase the titers of CFTR cDNA-containing retroviral vectors are described. Finally, methods are outlined for evaluating the phenotypic correction of the chloride transport defect.

1.1. Principles of Ion Transport in CF

The airway epithelium is a semipermeable barrier composed of surface epithelial cells together with the tight junctions that join them at their apical borders, and submucosal glands that secrete liquid and mucins into the airway lumen. These components play an important role in lung defense through secretion of airway surface liquid and active mucociliary clearance that acts to entrap and clear inhaled foreign substances. The regulation of the volume and composition of airway surface liquids is achieved by active ion transport.

In human airway epithelia under resting conditions, Na⁺ is the major ion flux across the airway epithelium. Sodium is absorbed across the epithelium in a two-step process that involves passive entry into the cell down an electrochemical gradient through conductive pathways or channels in the apical membrane followed by extrusion across the basolateral membrane by the activity of the Na⁺-K⁺-ATPase. The entry of Na⁺ into the airways is inhibited by the Na⁺ channel blocker, amiloride. Airway epithelia also possess the potential to add salt and water to the airway surface liquid through the secretion of Cl⁻. Chloride transport across the airway epithelium is complex and involves Cl⁻ entry across the basolateral membrane, electrochemical forces for Cl⁻ flow across the apical cell membrane, and apical membrane Cl⁻ permeabilities. Chloride may enter the cell from the submucosal compartment through the Na⁺-K⁺-2Cl⁻ cotransporter. When an electrochemical driving force exists, Cl⁻ may exit the apical surface of the cell and enter the airway surface liquid through either a cAMP-mediated Cl⁻ channel (CFTR) or, alternatively, through an alternative Cl⁻ conductive pathway that appears to be regulated by Ca²⁺-mediated agonists. Experimentally, a driving force for apical Cl⁻ exit can be created by blocking Na⁺ absorption with amiloride, which hyperpolarizes the apical membrane, and by removing Cl⁻ from the luminal surface of cells, which produces a chemical gradient.
In CF, Na⁺ absorption is raised two- to threefold over that measured in normal airway epithelia (4). This appears to be caused by an increased open probability of the apical Na⁺ channels (5) and is accompanied by an increase in the Na⁺-K⁺-ATPase number and/or activity (6). Moreover, CF is characterized by the absence of a cAMP-activated apical membrane Cl⁻ channel (i.e., CFTR). The localization of rate-limiting Na⁺ and Cl⁻ conductances and of normal CFTR in the apical cell membrane is probably important physiologically, and suggests that polarized epithelia may be essential to evaluate the cellular physiology that causes CF.

Because regulation of airway surface liquid composition involves the regulation of the movement of ions across the airways, polarized airway epithelial tissues generate a transepithelial resistance (Rt), a transepithelial potential difference (Vt), and a transepithelial current called the short circuit current (Isc) that can be monitored electrically. These measurements can be used to assess the phenotypic correction of the ion transport defect in polarized CF epithelial cells following gene transfer of a normal CFTR cDNA.

2. Materials

2.1. Primary Culture of Human Airway Epithelial Cells

1. F12 + 7X growth medium for primary airway epithelial cells (see Note 1): Medium F12 with glutamine and HCO₃⁻ (Life Technologies, Gaithersburg, MD) supplemented with 7 hormones and growth factors (Table 1). Penicillin (50 U/mL), streptomycin (50 µg/mL), and gentamicin (40 µg/mL) are added as antibiotics.

2. Joklik’s Calcium-free and Magnesium-free Minimum Essential Medium (JMEM) (Life Technologies).

3. Phosphate-buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 2.16 g Na₂HPO₄ · 7H₂O, and 0.2 g KH₂PO₄/L.

4. 3T3 fibroblast-conditioned medium: Grow 3T3 mouse embryo fibroblasts (American Type Culture Collection, Rockville, MD) in Dulbecco’s Modified Eagle’s Medium (DMEM, Life Technologies) containing 10% fetal bovine serum (FBS). When cells become 90% confluent, change the medium to DMEM containing 2% FBS (0.64 mL/cm²). After 3 d, collect conditioned medium, filter sterilize, and store at 4°C. The conditioned medium may be used within 3 wk, or stored at −20°C for up to 6 mo.

5. 1.0 mg/mL protease Type XIV (Sigma, St. Louis, MO) plus 0.1 mg/mL deoxyribonuclease I (Sigma) in JMEM (0.2-µm filter sterilized).

6. 0.4% trypan blue in PBS (Sigma).

7. Collagen-coated matrix supports (CMS) (see Note 2): These matrices are cast on a beveled orifice (4.5-mm diameter) in the bottom of a 2.5-cm diameter polycarbonate cup and can be made by the following previously published protocol that has been modified slightly (7)

   a. Dissolve type III calf skin collagen (Sigma) in 0.2% acetic acid at 15 mg/mL.
   b. Add 0.5 vol of 25% glutaraldehyde and cool in an ice bath until jelling begins.
<table>
<thead>
<tr>
<th>Additive</th>
<th>Stock preparation</th>
<th>Stock concentration</th>
<th>Stock storage temperature</th>
<th>Stock expires</th>
<th>Final concentration in growth medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>20 mg/4 mL H₂O</td>
<td>5 mg/mL</td>
<td>-20°C</td>
<td>3 mo</td>
<td>10 µg/mL</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>(1) 50 mg/13.8 mL ethanol</td>
<td>10⁻²M</td>
<td>-20°C</td>
<td>1 yr</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2) dilute 1:10 with PBS</td>
<td>10⁻³M</td>
<td>-20°C</td>
<td>3 mo</td>
<td>10⁻⁶M</td>
</tr>
<tr>
<td>ECGS</td>
<td>15.0 mg/4 mL H₂O</td>
<td>3.75 mg/mL</td>
<td>-20°C</td>
<td>1 mo</td>
<td>3.75 µg/mL</td>
</tr>
<tr>
<td>EGF</td>
<td>100 µg/4 mL H₂O</td>
<td>25 µg/mL</td>
<td>-20°C</td>
<td>3 mo</td>
<td>25 ng/mL</td>
</tr>
<tr>
<td>Triiodothyronine</td>
<td>(1) 20 mg/5 mL 0.2M NaOH</td>
<td>3 mM</td>
<td>-20°C</td>
<td>1 yr</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2) add 5 mL H₂O</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3) dilute 1:100 in H₂O</td>
<td>3 x 10⁻⁵M</td>
<td>-20°C</td>
<td>3 mo</td>
<td>3 x 10⁻⁸M</td>
</tr>
<tr>
<td>Transferrin</td>
<td>10 mg/4 mL H₂O</td>
<td>25 mg/mL</td>
<td>-20°C</td>
<td>3 mo</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td>Cholera toxin</td>
<td>0.5 mg/5 mL H₂O</td>
<td>100 µg/mL</td>
<td>-4°C</td>
<td>1 yr</td>
<td>10 ng/mL</td>
</tr>
</tbody>
</table>

*a* Abbreviations: ECGS, endothelial cell growth supplement, EGF, epidermal growth factor

*b* All additives can be obtained from Collaborative Research (Lexington, MA) except cholera toxin, which can be obtained from Sigma (St Louis, MO).

*c* Stocks do not need filtering if handled with sterile technique.
c. Apply a bead of collagen/glutaraldehyde around the orifice of the inverted polycarbonate, and drag mixture across the orifice with a Pasteur pipet, forming a thin sheet.

d. Dry in air (3–4 h).

e. Apply a second coat of collagen (with glutaraldehyde) over the bottom surface of the matrix. Dry overnight.

f. Apply a third coat of collagen (without glutaraldehyde) to the inside surface of the matrix. Dry overnight.

g. Rinse four times with 20 mM NaHCO3 in 130 mM NaCl. Dry overnight.

h. Coat inside surface with 30 μL calfskin collagen (3 mg/mL, Vitrogen, Celtrix Labs, Palo Alto, CA). Dry overnight.

i. Sterilize unit in 70% ethanol for 30 min. Rinse in sterile culture medium. Incubate overnight at 37°C in F12 + 7X growth medium to test for sterility.

j. CMS dishes can be stored in PBS at 4°C for up to 1 mo.

2.2. Gene Transfer Using Retroviral Vectors

1. Retroviral vector producing cells, produced according to the protocols as described previously (8,9).

2. Polybrene (Sigma) stock of 4 mg/mL and sterilized by passing through a 0.2-μm syringe filter.

3. Sodium butyrate (Sigma) stock of 500 mM and sterilized by passing through a 0.2-μm syringe filter.

4. Centrifugal concentrators: Either 300K Macrosep (Filtron Technology Corporation, Northborough, MA) or Centriprep-100 (Amicon, Danvers, MA) concentrator devices have been used successfully for concentrating retroviral vectors.

5. Ultracentrifuge tubes: We use conical-shaped polyallomer centrifuge tubes for the SW 28 rotor (Koncal, Beckman Instruments, Palo Alto, CA) that are ideal for pelleting virus to a small area. Conical-shaped tubes must be used with adapters (Beckman Instruments) that are placed in the bottom of the rotor and support the conical bottom of the tubes during centrifugation.

6. Disposable syringe filters: Syringe filters combining a prefiltration and final filtration in one step (e.g., Uniflo Plus 0.2 μm, Schleicher & Schuell, Keene, NH) allow the filtration of ~30 mL of virus-containing medium before it is necessary to change filters.

7. Hank’s Balanced Salt Solution (HBSS): 0.4 g KCl, 0.06 g KH2PO4, 8 g NaCl, 0.048 g Na2HPO4, and 1 g D-glucose/L.

2.3. Measurement of CFTR Function

1. Krebs bicarbonate Ringer’s (KBR) solution, pH 7.4, with the following constituents in mmol/L: Na+ 140, K+ 5.2, Cl− 120, Ca2+ 1.25, HCO3− 25, Mg2+ 1.25, HPO42− 2.4, H2PO4− 0.4, and D-glucose 5.0. Stock solutions of reagents in distilled water are prepared as follows: K2HPO4 8.36 g/L, KH2PO4 1.09 g/L, NaCl 134.41 g/L, NaHCO3 42.01 g/L. Fifty milliliters of each stock are added to a flask and brought up to 800 mL with distilled H2O. The solution is gassed by bubbling 95% O2/5% CO2 into the solution for 10–15 min to adjust the pH from
8.5–7.4. Fifty milliliters of a stock solution of 4.88 g/L MgCl₂·6H₂O and 2.66 g/L CaCl₂ (anhydrous) is then added followed by 0.9 g D-glucose dissolved in 10 mL water. (The presence of an insoluble precipitate after addition of the MgCl₂ and CaCl₂ means that the pH was too alkaline owing to insufficient gassing.) The solution is then brought up to 1 L with distilled water.

2. Cl⁻-free Ringer’s solution, pH 7.4, with the following constituents in mmol/L: Na⁺ 140, K⁺ 5.2, gluconate 120, Ca²⁺ 2.5, HCO₃⁻ 25, Mg²⁺ 1.2, HPO₄²⁻ 2.4, H₂PO₄⁻ 0.4, SO₄²⁻ 1.2, and D-glucose 5.0. To prepare 1 L, add 50 mL each of the K₂HPO₄, KH₂PO₄, and NaHCO₃ stocks from above plus 200 mL of a 125.4 g/L Na gluconate stock to a flask. Bring to 800 mL with distilled water and gas with 95% O₂/5% CO₂ for 10–15 min. Add 50 mL of 10.66 g/L calcium gluconate with 5.92 g/L MgSO₄·7H₂O and 0.9 g D-glucose dissolved in 10 mL water. Bring up to 1 L with distilled water.

3. Forskolin (Sigma), a 10⁻²M stock of 4.1 mg/mL in 70% ethanol.
4. Amiloride (Sigma), a 10⁻²M stock of 2.66 mg/mL in water.
5. Current voltage clamps, Model VCC600, Physiologic Instruments, San Diego, CA.
6. Epithelial V/Q meter (EVOM), World Precision Instruments, Sarasota, FL.
7. Lucite Ussing chambers: These are available from a variety of sources, including World Precision Instruments, or they can be made in your local physics shop.
8. Twin perfusion columns, glass: These are available from World Precision Instruments, a variety of other companies, and can also be made in your local glass shop.

3. Methods

3.1. Isolation and Primary Culture of Human Airway Epithelial Cells

3.1.1. Tissue Procurement and Transport

1. Obtain excess excised nasal or bronchial tissues from fresh surgical specimens (e.g., nasal polyps; see Notes 3–5).
2. Transport to laboratory in chilled (4°C) JMEM solution.
3. Remove and discard loose connective tissue by blunt and sharp dissection.
4. Incubate in excess volume (30–60 mL/5 g tissue) of chilled (4°C) JMEM containing penicillin (50 U/mL), streptomycin (50 mg/mL), and gentamicin (40 mg/mL) for 2–24 h.

3.1.2. Cell Isolation

1. Rinse tissue with fresh JMEM plus antibiotics, and incubate in 5 mL 1.0 mg/mL protease Type XIV plus 0.1 mg/mL deoxyribonuclease I in JMEM at 4°C for 16–48 h, agitating gently 4–6 times (see Note 6).
2. Add fetal bovine serum to 10% to neutralize the protease.
3. Remove tissue pieces, pellet cell suspension (500g for 5 min).
4. Wash cells with JMEM containing 10% FBS and pellet at 500g for 5 min.
5. Stain aliquot of cells with trypan blue solution, and count viable and dead cells in a hemacytometer.
6. Resuspend cells in growth medium to desired volume (see Section 3.1.3.).
3.1.3. Primary Culture

The decision to grow primary airway epithelial cells on plastic or on collagen supports will be influenced by the purpose of the experiment. For testing the efficiencies of gene transfer vectors or for using an anion efflux assay to measure CFTR function, culturing cells in plastic tissue culture dishes will suffice. For experiments requiring polarized sheets for measuring the vectorial transport of ions, it will be necessary to culture cells on collagen supports.

1. Plastic: Plate cells in F12 + 7X growth medium in 35-mm dishes (or six-well plates) at $2.5 \times 10^5$ viable cells/dish. After 24 h, wash cells with growth medium, and replace medium every 2–3 d. The plating efficiency should be 20–40%.
2. Collagen supports: Plate cells at $1-5 \times 10^6$ cells/cm$^2$ (see Note 7). Rinse gently after 24–48 h, then replace medium every 2–3 d. The plating efficiency should be about 50–80%.

3.2. Gene Transfer Using Retroviral Vectors

Primary human airway epithelial cells are a good host for retroviral vectors containing either the amphotropic envelope or pseudotyped with the gibbon ape leukemia virus (GALV) envelope. We have observed the highest gene transfer efficiencies (60–80% of primary cells transduced after a single infection) with retrovirus vectors containing the GALV envelope (10); however, it is not uncommon to transduce 20–40% of primary cells with amphotropic vectors (10,11).

1. Plate cells in plastic tissue culture dishes or on collagen supports as described in Section 3.1.3.
2. At 72 h after plating, infect cells by replacing medium with 0.5–1 mL retroviral vector. Add polybrene to 8 µg/mL and return cells to incubator.
3. After 2 h, remove virus and replace with fresh growth medium. Analyze cells for expression 48 h after infection.

3.3. Boosting Titers of Retroviral Vectors Containing CFTR cDNA

A problem often encountered by investigators is that the titer of recombinant retroviruses is inadequate for gene transfer applications. For retrovirus vectors containing a normal CFTR cDNA, the preparation of high-titer packaging cell lines is further confounded by the observation that high level expression of CFTR affects the growth rate and viability of many cell types including NIH 3T3 fibroblasts (12), the parental cell type of many widely used packaging cell lines. Here we describe methods for inducing the increased production of retrovirus vectors by treating producer cell lines with sodium butyrate and concentrating retrovirus vectors using centrifugal filtration or by pelleting in the ultracentrifuge. By using these two approaches in tandem, it is possible to increase viral titers 100-fold to 1000-fold.
3.3.1 Boosting Titer by Sodium Butyrate Treatment

We have used this method to increase titers of amphotropic retrovirus vectors produced by PA317 cells (13) and to increase the production of GALV-pseudotyped vectors produced by PG13 cells (14). For a given clonal packaging cell line, preliminary titration of sodium butyrate (from 0.5–30 mM) should be done to find the concentration giving the optimum enhancement of vector production. Depending on the vector and the particular producer clone (see Note 8), sodium butyrate treatment may enhance vector production from twofold to >1000-fold (15).

1. Plate the virus producer cells in tissue culture dishes and allow the cells to grow until they have just reached confluence.
2. Remove the medium from the cells and replace with fresh growth medium containing sodium butyrate. Place cells back into CO₂ incubator.
3. After 20–24 h of sodium butyrate treatment at 37°C, or after 48 h of treatment at 32°C (see Note 9), remove the supernatant containing the virus and pass through a 0.2-μm syringe filter to remove cells and cell debris. Alternatively, cells can be selectively pelleted from medium by centrifugation at 5000g for 10 min.
4. The virus can be stored indefinitely in aliquots at -70°C, or it can be concentrated immediately by the methods described in Sections 3.3.2. or 3.3.3.

3.3.2. Concentrating Retroviral Vectors by Centrifugal Filtration

This procedure is a relatively gentle method for concentrating retroviral vectors approx 30- to 50-fold without significant loss of infectivity.

1. Prerinse filters (100- to 300-μm pore size) before use by centrifuging 15 mL deionized, sterile H₂O through filtration device for 20 min. Discard filtrate and any remaining retentate. If filtration device is not going to be used immediately, keep filter membrane wet with H₂O. Decant H₂O just prior to use. If desired, the filtration unit can be presterilized prior to use by following the manufacturer’s instructions.
2. Add 15 mL virus (0.2-μm filtered) to the appropriate chamber of the filtration device. If virus has been frozen after harvesting from the producer cells, it is necessary to pass through a 0.2-μm filter to remove any serum protein denatured by the freeze-thaw process.
3. Centrifuge in a fixed angle rotor at 500g at 4°C. Every 30 min, stop the centrifugation and remove the filtrate until the volume of the retentate containing the virus reaches the desired reduction in volume.
4. Remove particulate matter from the virus by passing the retentate through a 0.2-μm syringe filter (prewetted with H₂O). Store virus in aliquots at -70°C.
3.3.3. Concentrating Retroviral Vectors by Pelleting in an Ultracentrifuge

The successful application of this method appears to be dependent on the stability of the viral envelope glycoproteins. Murine retroviral vectors with the ecotropic envelope surface glycoprotein or pseudotyped with the GALV or vesicular stomatitis virus G (16) envelope glycoproteins can be pelleted with significant (>25–90%) recovery of total infectivity. In contrast, pelleting vectors with the amphotropic envelope results in poor recovery (<15%). The following method has been used with the Beckman SW 28 rotor using the conically shaped Konical tubes (30-mL capacity) to pellet virus, but should be adaptable for use with other rotors and tubes.

1. Prerinse polyallomer ultracentrifuge tubes successively with ~20-mL aliquots of acetone (three times), 95% ethanol (twice), and endotoxin-free, double-distilled H₂O (three times). Allow tubes to drain in tissue culture hood.
2. Fill centrifuge tubes with virus (0.2-μm filtered) collected from the vector producer cells. If the virus has been frozen, denatured protein can be removed from the thawed virus by filtering using a syringe filter. If relatively large volumes (>50 mL) of virus are to be processed, it is advisable to use a syringe filter with a prefILTER (e.g., Uniflo Plus).
3. Carefully place tubes into rotor buckets. (If the Beckman Konical tubes are used, be sure to use special adapters to prevent tube collapse.)
4. Pellet the virus using a swinging bucket rotor (e.g., SW 28) in an ultracentrifuge at 100,000g for 2 h at 4°C.
5. Decant supernatant and allow tubes to drain. Remove excess liquid from sides of tube above the pellet with Kimwipes or other lintless laboratory wipers.
6. Suspend virus in a small volume of HBSS and store in aliquots at -70°C. Alternatively, suspend virus in growth medium and use for immediate gene transfer as described in Section 3.2.

3.4. Measurement of CFTR Function

A variety of methods are available for measuring the function of CFTR. Most of these assay systems focus on the measurement of a cAMP-mediated Cl⁻ secretory response. Some of the assays also permit the evaluation of basal Na⁺ transport and Cl⁻ secretory function.

3.4.1. Efflux Assays

The advantages of efflux assays are that they do not require an intact epithelium and they can be easily performed on cells grown in plastic dishes. These assays measure the rate of loss of Cl⁻ from corrected and/or normal cells as compared to CF epithelial cells. The cAMP-mediated agonists such as forskolin or other β-agonists may be added to more critically evaluate the cAMP-medi-
ated Cl\(^-\) secretory response. Corrected or normal airway epithelial cells should exhibit a significantly increased rate of Cl\(^-\) efflux compared to CF cells.

Two different kinds of anion efflux assays are currently being employed: those based on radioactive isotopes, and those involving the use of ion-selective electrodes (17). The radioisotopic assays use either \(^{36}\)Cl\(^-\) or \(^{125}\)I\(^-\). Published methods for efflux assays have generally been used with nonairway epithelia, and may require adaptations for airway epithelia. The \(^{36}\)Cl\(^-\) efflux assay is described here (see Note 10):

1. Grow cells in 35-mm plastic tissue culture dishes to about 70–90% confluence. For cells infected with retroviral vectors, analyze cells 48 h after infection.
2. Load cells with radioactive chloride by replacing the growth medium with 1 mL KBR, pH 7.4, containing 5.0 μCi \(^{36}\)Cl\(^-\) (specific activity > 3 mCi/g Cl\(^-\)). Incubate in tissue culture incubator for 2 h at 37°C.
3. Wash cells four times rapidly (30–45 s/wash) with 1.0 mL KBR at room temperature.
4. After last wash, add and remove sequentially at 1 min intervals 1-mL aliquots of isotope-free Cl\(^-\)-free Ringer’s solution with 10\(^{-4}\)M amiloride at room temperature. Carry out assay for 10 min. Save each sample in a 20-mL liquid scintillation vial.
5. To assess CAMP-mediated Cl\(^-\) permeability, add 10 μL 10\(^{-3}\)M forskolin to the 1-mL aliquots of sampling buffer (Cl\(^-\)-free Ringer’s solution with 10\(^{-4}\)M amiloride) at the 3-min time point.
6. At the end of the sampling period, extract the radioisotope in the cell layer with 2 × 500 μL washes of 0.1% sodium dodecyl sulfate. Combine these washes in a 20-mL liquid scintillation vial.
7. Add 10 mL of an appropriate liquid scintillation counting fluid to each sample. Determine the amount of \(^{36}\)Cl\(^-\) in the efflux aliquots and cells by liquid-scintillation counting. Calculate the total radioactivity loaded into cells from the sum of the counts of each individual efflux time interval plus the counts remaining in the cells at the end of the sampling period.
8. Plot efflux curves as the percent of counts remaining in the cells vs time. Rate constants, if desired, may be determined by fitting efflux curves to exponential functions using a standard statistics computer program.

3.4.2. Bioelectric Characterization of Ion Transport in Epithelial Sheets

The ion transport properties that characterize cystic fibrosis are dependent on polarized epithelial cells. Thus, the advantage of using assays to measure ion transport across epithelial sheets grown on permeable collagen matrix supports (CMS) is that many in vivo properties of an intact epithelium are preserved. The CMS cultures develop tight junctions and form a polarized monolayer with the basolateral surface of the plasma membrane towards the filter and the apical surface toward the culture medium. Ion channels often
show specificity in localizing to the apical membrane (e.g., CFTR) or basolateral membrane and this property is retained in CMS cultures (9,18). Application of electrophysiological techniques to CMS cultures provides an opportunity for detailed studies of the role of apical and basolateral membrane channels in transepithelial ion movement. Ussing chambers allow separation of the apical solution from the basolateral solution. Therefore, the movement of ions from one bath to the other must involve either transcellular flow or flow through the paracellular path of the epithelial sheet. The current required to clamp the $V_i$ to zero, the short circuit current ($I_{sc}$), is monitored continuously except for measurements of the spontaneous $V_i$ across the epithelial sheet at about 5-min intervals. The transepithelial resistance of cultured airway epithelial preparations is dominated by the paracellular path such that changes in apical membrane Cl$^-$ permeability have minimal effect on transepithelial resistance. We describe here methodology that can be used to differentiate between normal and CF airway epithelia, and to assess the ion transport properties of phenotypically "corrected" CF cells following gene transfer of a normal CFTR cDNA.

1. Cultures growing on CMS are transduced with the gene transfer vector on d 3 as described in Section 3.2.
2. On d 4 of culture, the culture medium is changed to a mixture of 50% F12 + 7X and 50% 3T3 conditioned medium. This growth medium formulation helps in the formation of epithelial sheets with tight junctions (see Note 11).
3. Measure $V_i$ and $R_i$ beginning on d 3–4 in cultures using an epithelial V/Ω meter (EVOM). The EVOM electrodes should be sterilized before use by soaking in 70% ethanol and equilibrated in growth medium prior to use. All measurements should be made in a tissue culture hood at room temperature. $R_i$ should also be measured across a blank CMS and subtracted from $R_i$ to yield the tissue resistance to be used for calculation of current. When the culture has fully differentiated into an epithelial sheet with tight junctions (d 6–7 in culture), the $V_i$ and $R_i$ measurements will reach plateau values corresponding to a $V_i \approx 10$ mV for normal cells and ~20–30 mV for CF cells with $R_i = 300–400 \, \Omega \cdot \text{cm}^2$. If cell lines are used instead of primary cells, the $V_i$ is generally lower, although $R_i$ tends to be similar to that of primary cultures.
4. Mount cultures in Ussing chambers containing bilateral KBR (pH 7.4) and interface to electrometers. During the experiment, the buffer in each chamber is continuously gassed by bubbling with a mixture of 95% O$_2$/5% CO$_2$.
5. Place current-voltage clamp in voltage clamp mode to monitor the basal short circuit current ($I_{sc}$) and begin recording data continuously on chart recorder. Using the pulse circuit modulator, pass a 2–5 mV pulse every 10–15 s. This allows determination of the change in $R_i$ and can be used to calculate $V_i$ using Ohm's law. Alternatively, switch to open circuit momentarily to read $V_i$ directly. Continue monitoring the basal $I_{sc}$ until it stabilizes (5–10 min).
6. Add 10 μL/mL 10⁻²M amiloride to the luminal bath (the solution bathing the apical side of the epithelial sheet away from the filter). Continue to take measurements until the response stabilizes (about 5 min).

7. Drain the luminal bath and replace with a solution of Cl⁻-free Ringer’s solution, pH 7.4, containing 10⁻⁴M amiloride. When the Iₛₙ stabilizes (about 10–30 min), add 1 μL/mL of 10⁻²M forskolin. Continue taking measurements until a steady state level is reached (about 3–5 min).

3.4.3. Interpretation of Transepithelial Bioelectric Data

3.4.3.1 Basal Na⁺ Transport

The Iₛₙ when the epithelial sheets are mounted in KBR solution represents basal Na⁺ absorption. This has been shown with both cultured and excised human airway epithelia preparations (4,19). In CF, the Iₛₙ is increased two- to threefold over that measured in normal airway epithelia.

3.4.3.2 Basal Cl⁻ Permeability

In normal or corrected airway epithelia, the Na⁺-channel blocker amiloride abolishes the basal Na⁺ absorption and induces Cl⁻ secretion at a rate that is proportional, in part, to the apical membrane Cl⁻ conductance. However, because the apical membrane Cl⁻ conductance is absent in CF, no significant Cl⁻ secretion is induced by amiloride. Replacement of the solution in the luminal bath with a Cl⁻-free solution causes a change in Vₜ, which reflects, in part, the Cl⁻ permselectivity of the apical membrane. In CF, this change tends to be small, whereas in normal or corrected CF airway epithelium, the change in Vₜ with Cl⁻-free substitutions is markedly increased (9,20–23). The small change that does occur in CF reflects movement of Cl⁻ through the paracellular path, whereas the change that occurs in normal or corrected tissues reflects both paracellular movement flow and the movement of Cl⁻ through CFTR Cl⁻ channels in the apical membrane (20,21,24).

3.4.3.3. cAMP-Mediated Cl⁻ Permeability

The change in Iₛₙ in response to cAMP-agonists is used to assess regulation of the apical membrane Cl⁻ conductance by CFTR. This response tends to be negligible in CF, whereas it tends to be significantly greater in normal or corrected airway epithelial cells (9,20–23).

4. Notes

1. Growth factor supplements: Insulin and ECGS are essential for proliferation. The other factors have smaller effects on proliferation rates, and may be titrated to meet individual needs.

2. Permeable tissue culture substrates: Commercially prepared substrates made of polycarbonate or polyester, and with various coatings are available from Costar (Cambridge, MA), Millipore (Bedford, MA), and other manufacturers.
3. Research use of human tissues: Research involving human subjects must be reviewed and approved by the local Institutional Review Board (IRB). The University of North Carolina at Chapel Hill IRB has determined that the research use of *excess excised tissues* that were removed for standard clinical indications is authorized by the consent obtained in the IRB approved Consent for Operation Form, and that additional consent is not required.

4. Application to other species: These techniques can be applied, with minor variations, to nasal and airway tissues from mice, dogs, and other laboratory animals (23,25,26).

5. Airway epithelial cell lines: Immortalized airway epithelial cell lines have been generated from primary cultures with simian virus 40 and human papilloma virus genes (18,27–32). These cell lines have increased growth capability and variable retention of differentiated phenotypic properties.

6. Cell yield and viability: Epithelial cell yield increases and viability decreases by increasing protease exposure times to 72 h. The percentage of ciliated cells decreases with longer incubation times.

7. Plating on permeable supports: Epithelial cell attachment to collagen-coated supports can be maximized by plating epithelial cells in a minimal volume of medium, and adjusting the volume of medium in the outer well to maintain a small hydrostatic gradient opposing the cells to the surface of the support. Hydration is maintained by a humidified incubator and by adding medium to adjacent wells in a multiwell culture dish.

8. Sodium butyrate: The effect of sodium butyrate on increasing vector production appears to be by increasing steady-state levels of RNA transcribed from the retroviral LTR sequence (15). We have not detected the induction of replication-competent retroviruses in vector-producing cultures after sodium butyrate treatment. For reasons incompletely understood, there is a vector construct dependency on the extent of induction by sodium butyrate. For example, production of the CFTR-containing LCFSN vector (9) is increased 20-fold to 1000-fold, whereas production of vectors containing other inserts is increased only up to about tenfold (15).

9. Temperature of retroviral vector production: A previous report suggests that increased titers can be achieved by incubating packaging cells at 32°C and collecting virus after 48 h (33). We have confirmed this result for many but not all producer cell lines. In addition, at least for CFTR-containing vectors, the combination of lowered temperature and sodium butyrate treatment often results in a synergistic enhancement of retroviral vector production (15).

10. Alternate efflux assay: Another method of performing anion efflux assays is to use an iodide-sensitive electrode. The technique is similar to that described in Section 3.4.1., but instead of using a radioisotope, the cells are loaded with non-radioactive NaI and the rate of loss of iodide from the cells is measured with an iodide-sensitive electrode at 1-min intervals.

11. Differentiation inducing conditions: Polarized morphology, ion transport, and other differentiation functions can be induced by combinations of the following
conditions: plating at high density (1–5 x 10^6 cells/cm², depending on cell type); adding a hydrated collagen gel (0.8 mL of 3 mg/mL acid solubilized collagen, 0.2 mL of 5X concentrated F12 medium, and sufficient NaOH to alkalinize to pH 8.0) to the filter followed by incubation at 37°C for 30 min before plating cells, exposing the apical surface of cells to humidified air/CO₂ (rather than medium) after attachment, and addition of retinoic acid, 10⁻⁷–10⁻⁶M to the culture medium.

12. Quality of CMS tissue preparations: Cultures that are not confluent or fail to develop a resistance (e.g., cultures that contain a significant proportion of cell types other than epithelial cells) are excluded from study. Cultures that have Vᵢ ≤ -0.5 mV or Rₑ < 50 Ω · cm² generally tolerate mounting in Ussing chambers poorly and may have to be excluded. Exclusion criteria should be established before initiating the experiment to prevent bias in data analysis.

References


Methods for Adenovirus-Mediated Gene Transfer to Airway Epithelium

John F. Engelhardt

1. Introduction

Recombinant adenoviral vectors have proven to be extremely efficient tools for transferring genes into multiple organ systems including the lung (1-5), liver (6-10), muscle (11,12), joint (13), vasculature (14,15), and central nervous system (16-18). The lung presents unique advantages as a target organ for the evaluation of these vectors as tools for in vivo gene therapy owing to the accessibility of target cells by direct instillation or inhalation of recombinant virus in the airway. Multiple therapeutic applications of gene therapy to the lung include both inherited disorders such as cystic fibrosis (CF) and α1-antitrypsin deficiency, as well as acquired disorders such as bronchitis, asthma, emphysema, and respiratory malignancies. The most widely studied application to date has involved the analysis of recombinant adenoviruses for gene therapy of CF lung disease (1-5). To this end, multiple phase I clinical trials are now underway evaluating the safety of recombinant adenoviral vectors for gene therapy of cystic fibrosis lung disease (19). This clinical application of these vectors in CF lung disease presents the largest comprehensive effort of in vivo gene therapy to date. Such a substantial effort has offered the advantage of multiple preclinical studies evaluating delivery of adenoviral vectors to the lung in numerous animal models such as the cotton rat (4,5,20), human bronchial xenografts (1), mouse (21), and nonhuman primates (2,3,22,23).

Cystic fibrosis is the most common autosomal recessive disease in the Caucasian population, affecting 1 in 2500 births each year (24). Although multiple organs are affected in this disease, the most severe and life-threatening pathology occurs in the lung and results in production of abnormally viscous mucus, poor mucociliary clearance, chronic bacterial infections, bronchiectasis, and...
eventually pulmonary failure and death. The cloning of the gene defective in CF (CFTR, the cystic fibrosis transmembrane conductance regulator) has allowed for the identification of the cellular defect and has made possible the application of gene replacement as a potential therapy for lung disease (25–27). The cellular targets for gene therapy of CF lung disease have traditionally been thought to reside in the small airways, as inferred by the fact that this region is most severely affected (24). Recent studies localizing CFTR mRNA and protein have demonstrated that multiple cellular targets throughout the lung exist, including a subpopulation of cells in the submucosal glands, bronchioles, and alveolar regions (28a,28b). The pathophysiologic significance of each of these cellular and anatomical target regions based on expression studies is unknown. However, current therapeutic approaches delivering recombinant virus directly to the airway will most likely exclude gene transfer to submucosal gland regions.

The airways of the lung can be grossly divided into two regions, the conducting and respiratory epithelium. The cell types that compose the conducting airway epithelium within the cartilaginous bronchi and large bronchioles include a heterogeneous combination of cell types including basal, goblet, intermediate, and ciliated cells (29). Although it is generally accepted that both goblet and basal cells have the capacity for self renewal, the pleuripotent characteristics of these cell types to give rise to ciliated and intermediate cells is currently debated (30–32). More distal regions of the conducting airway epithelium include the smaller bronchioles that are composed of at least two cell types, Clara and ciliated cells (33). Studies localizing CFTR to a subset of nonciliated cells in this region have suggested that the target cell in the distal conducting airways may in fact be a subset of Clara cells (28a). The progenitor cell of this region has traditionally been thought to be the Clara cell (33). The most distal regions of the lung that are composed of the respiratory epithelium may also be an important target for gene therapy of CF as suggested by recent studies localizing CFTR mRNA and protein (28a). This region is composed of type I and II alveolar cells as well as alveolar macrophages (33). The progenitor cell of this region is generally accepted to be type II alveolar cells (33). Strategies for prolonged gene therapy to the lung in CF will likely need to target progenitor cells in the airways that give rise to CFTR-expressing cellular targets. Because the turnover of airway epithelial cells in the airway is relatively long in the nondisease state (31), alternative efficient strategies targeting differentiated CFTR-expressing cells directly, may also provide substantial lengths of correction.

Current recombinant adenoviral constructs have been based on the Ad5 and Ad2 serotypes in which transgenes are inserted into the E1 deleted region. Deletion of E1 sequences serves two functions in the construction of recombi-
nant adenoviral vectors, including the crippling of adenoviral replication and the creation of space for transgene insertions. Because of the limited packaging size of adenoviral DNA (14), the application of recombinant adenoviral vectors for gene therapy of CF in which the CFTR cDNA is 4.7 kb, has created the need for further deletion of non-E1 regions of the adenovirus. Two approaches including the deletion of E3 sequences or E4 sequences not needed for replication of the virus have been used to circumvent problems associated with this large cDNA insert.

Multiple animal models including the cotton rat (4,5,20,23), mouse, human bronchial xenografts (1), and nonhuman primates (2,3,23) have been used for evaluating the efficiency of recombinant adenoviral gene transfer to the lung epithelium. Each animal model possesses several advantages in the analysis of host-vector interactions and efficiency of gene transfer. The most commonly used model includes the cotton rat, which has been shown to be permissive for adenoviral infection and provides a pathophysiologically relevant correlation to human adenoviral infections in the lung (35). In contrast, the mouse, traditionally thought to be less permissive for adenoviral infection, provides the added advantage of genetically defined mouse strains for the evaluation of host-vector inflammatory responses in the generation of more therapeutic viral vectors with increased persistence and decreased host inflammatory responses (21,36). The human bronchial xenograft, although immune incompetent with respect to T-cells, provides the ideal system for the analysis of cellular interactions between human epithelium and these vectors. This system has been useful in addressing the capacity of these vectors to replicate and express viral genes within the relevant target human airway epithelium (1). Lastly, nonhuman primates have proven to be useful animals in the safety and efficacy evaluation of recombinant adenoviral vectors within an animal model that is evolutionarily close to humans. In addition, this model has allowed for the evaluation of alternative delivery systems (i.e., delivery to a subsegment of the lung via bronchoscopy) that are not feasible in rodent models (2).

Several delivery methods have been used in the analyses of recombinant adenoviral vectors in animal models targeting airway epithelium, including: transtracheal instillation of virus by midline incision (4,5,20,23), selective administration of virus to subsegments of the lung by bronchoscopy (2), and nasal administration of virus (37,38). Analysis of in vivo delivery of these vectors to the airways of immune competent animal models has demonstrated efficient recombinant adenovirus-mediated gene transfer, which is transient (<21 d) and associated with mononuclear-predominant inflammatory infiltrates (20,21). Alternatively, immune incompetent (T-cell deficient athymic mice) animal models, including human bronchial xenografts grafted into nu/nu mice and nu/nu mouse lung, have demonstrated that extended persistence of
transgene expression (>100 d) can be achieved in the absence of inflammatory responses (21). These studies have set the stage for the improvement of these vectors for gene therapy applications by alteration of the adenoviral genomic components that are responsible for eliciting host cellular immune responses and the decline of transgene expressing cell in vivo (20,21). Hence, although the current applications of these vectors for gene therapy will require frequent repetitive doses, the future construction of second generation vectors has provided a renewed enthusiasm for the use of these vectors with prolonged transgene expression and minimal host inflammatory responses (20,21).

Critical to the evaluation of recombinant in vivo models of gene transfer are sensitive methods for the analysis of transgene expression. Hence, this review will also discuss the applications and methodologies of histochemical, immunocytochemical, and in situ hybridization localization techniques for the analysis of transgene-expressed proteins and mRNA (1,2). Such methodologies provide the foundation for the therapeutic assessment of efficacious gene transfer to the lung.

2. Materials

2.1. Solutions

1. Glycerol-BSA viral storage buffer: 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1% BSA, 50% glycerol. Filter sterile through a 0.2-μm filter and store at -20°C
2. Heavy CsCl: Add 42.23 g CsCl to 57.8 mL 10 mM Tris-HCl, pH 8.0 (final density of 1.45 g/mL).
3. Light CsCl: Add 22.39 g CsCl to 77.6 mL 10 mM Tris-HCl, pH 8.0 (final density of 1.20 g/mL).
4. X-gal staining solution: 1 mg/mL X-gal (5-bromo-4-chloro-3-indoly-β-D-galactopyranoside), 5 mM K₂Fe(CN)₆, 5 mM K₃Fe(CN)₆, 1 mM MgCl₂, in PBS, pH 7.4.
5. DepC treatment of water and plastic wear: 1 mL depC IS mixed with 1 L of H₂O and allowed to inactivate RNase for 20 h followed by autoclaving for 1 h.
6. Proteinase-K in situ solution: 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 10 μg/mL proteinase-K.
7. In situ prehybridization solution: 10 mM Tris-HCl, pH 8.0, 50% formamide, 2.5X Denhardts, 0.6M NaCl, 1 mM EDTA, 0.1% SDS, 500 μg/mL Escherichia coli tRNA, and 10 mM DTT.
8. In situ RNase A pretreatment buffer: 200 μg/mL RNase A in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA
9. In situ hybridization buffer: 1 × 10⁷ cpm/mL of sense or antisense radiolabeled probe in 10 mM Tris-HCl, pH 8.0, 50% formamide, 2.5X Denhardt's, 25% Dextran sulfate, 0.6M NaCl, 1 mM EDTA, 0.1% SDS, 500 μg/mL E. coli tRNA, and 10 mM DTT.
10. In situ RNase A posthybridization wash buffer: 20 μg/mL RNase A in 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA.
Adenovirus Transfer to Airway Epithelium

2.2. Antibodies

1. Goat anti-Ad3-FITC antibody: Chemicon (El Segundo, CA) #1056.
2. Rabbit anti-β-galactosidase antibody: 5'→3' Inc. (West Chester, PA) #5307-063100.
3. Goat antirabbit-FITC antibody: Boehringer Mannheim (Indianapolis, IN) #100825.

3. Methods

3.1. General Propagation and Purification of Adenovirus

The propagation of recombinant adenoviral vectors, in which transgenes are inserted into the E1 deleted region of the adenoviral genome, are performed in the complementing 293 cell line, which provides deleted E1 functions in trans by stably integrated E1a and E1b viral genes within the cellular genome. Because the purity and functional integrity of recombinant viral preparation are extremely crucial for applications of in vivo gene transfer, this review will briefly summarize methods used for the propagation and analysis of recombinant adenoviral stocks for application to the lung.

1. Seed 40 × 150 mm tissue culture dishes with 293 cells in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (PS).
2. Eighty percent confluent, 150-mm dishes of 293 cells are infected with purified stocks of virus at an MOI = 10 in DMEM/1%PS in the absence of FCS for 2 h, total volume of 10 mL. Since, on average, an 80% confluent, 150-mm dish of 293 cell consists of 5 × 10⁷ cells, we routinely infect with 5 × 10⁸ PFU/plate (see Note 1).
3. Two hours postinfection, 10 mL of 20% FCS/DMEM/1%PS are added to each plate and the infection is allowed to progress for 30–48 h. Plates should be considered ready to harvest when infected cells can be easily dislodged by tapping (see Note 2).
4. Infected cell suspensions are harvested and combined in 1000-mL centrifuge bottles, cooled to 4°C on ice, and pelleted by centrifugation at 3500g in a Beckman J6 centrifuge for 25 min at 4°C.
5. Supernatants are discarded and cell pellets resuspended in 20 mL of 10 mM Tris-HCl, pH 8.0, for hypotonic lysis followed by three rounds of freeze thawing in an ethanol dry ice and 37°C baths. Care should be taken to avoid extended periods at 37°C.
6. Crude lysates are then pelleted to remove cellular debris by centrifugation at 1500g for 25 min in a table-top centrifuge at 4°C.
7. The supernatants that contain crude viral lysates are carefully removed so as not to disturb the loose cellular pellet. To increase yields, the cellular pellet is resuspended in an additional 6 mL of cold 10 mM Tris-HCl, pH 8.0, and the centrifugation is repeated. At no time should the cellular/viral suspensions be vortexed, as this results in shearing of genomic DNA and complications in the CsCl purification.
Combined viral supernatants (18 mL) are loaded onto a double-stack CsCl step gradient consisting of a 10-mL heavy solution (solution 2) and a light (solution 3) CsCl cushion and centrifuged at 50,000g for 2 h in a Beckman SW28 rotor at 5°C.

The lower band of intact virus is removed, combined with an equal volume of 10 mM Tris-HCl, pH 8.0, loaded onto a second step gradient consisting of a 4-mL heavy solution (solution 2) and a light solution (solution 3) CsCl cushion, and centrifugated overnight for 16–18 h at 75,000g in an SW41 rotor at 5°C.

The lower band of virus is removed for preparation of desalting. Typical preparations starting with 40 150-mm plates of 293 cells should end with approx 0.5 mL of concentrated CsCl purified virus.

### 3.2. Desalting of Concentrated Viral Stocks for In Vivo Use

Desalting of concentrated viral stocks for preparation of in vivo delivery is a critical step in which the most concentrated stocks (>2 × 10^13 particles/mL) may precipitate depending on the method used. Precipitation of adenovirus will drastically lower the functional titer several logs. We have found that traditional methods of dialysis into PBS or buffered saline solutions increases the likelihood of precipitation of high titer stocks and, hence, we have adopted a method of gel-filtration desalting that is more rapid and avoids problems of variable precipitation encountered with dialysis.

1. 10-mL gel-filtration columns are constructed using typical 10-mL laboratory plastic pipets plugged with sterile glass wool and fitted with tubing to regulate flow. These columns are filled with a 50:50 slurry of autoclaved Sephadex G50 (medium grade) hydrated in PBS.
2. 0.5 mL of concentrated CsCl-banded virus is loaded onto gel-filtration columns, eluted in PBS, and the effluent virus (which is clearly visible as it migrates through the column as an opaque band) is collected in 250-μL aliquots.
3. The fractions composing the predominant visual peak are assessed for concentration of virus particles by OD_{260} and the most concentrated fractions combined.
4. Typical recoveries by this purification scheme are 70–90% with less than a two-fold dilution from the original sample.

Once virus has been purified, it should be used immediately for in vivo gene transfer experiments. A small sample should be diluted 1:5 with glycerol-BSA viral storage buffer (solution 1) and stored at −20°C for titering on 293 cells. Storage by this method for up to several weeks does not result in any detectable loss of viral titer.

### 3.3. Titering of Viral Stocks by Gene Expression

Crucial to the interpretation of in vivo gene transfer efficiency are accurate methods for titering of viral stocks for experimentation. Such titering should assess the relative fraction of virus capable of expressing transgene in refer-
ence to the total number of viral particles present in the sample as detected by OD\textsubscript{260} (where an OD\textsubscript{260} of 1 is equal to 10\textsuperscript{12} particles). The resultant ratio (conc. total particles)/(conc. functional particles), gives the necessary information for assessing the infectability of stocks used. Three methods of titering have traditionally been used for assessing the infectability of recombinant viral stocks including: PFU (plaque forming units), LFU (lacZ forming units), and FFU (fluorescent foci forming units). Since the readout of the PFU assay typically takes 7–9 d, we have attempted to develop assays based on the presence of viral protein or transgene expression in 293 indicator cells that can give titers within 20 h.

1. Subconfluent monolayers of 293 cells grown in 60-mm, six-well tissue culture dishes are infected with serial dilution of viral stocks made in DMEM/1%PS (ranging from \(10^{-4}\)–\(10^{-9}\)).
2. 1 mL of each dilution is placed in each well for 2 h.
3. Add 100 µL FCS to each well and incubate for an additional 18 h. Since the replication time of E1-deleted recombinant virus is approx 24 h, it is critical that the timing of infections not exceed 20 h (see Note 3).
4. For LFU assays, media from each well are aspirated and 0.5% glutaraldehyde in PBS are added gently to each well for 10 min.
5. Cells are then washed two times for 15 min in PBS containing 1 mM MgCl\textsubscript{2}.
6. LacZ transgene expression is then detected in the presence of X-gal staining solution (solution 4) at 37°C for 6–10 h.
7. Five 20X fields are quantitated for the total number of lacZ expressing cells and the total number of lacZ expressing cells in each well determined by the fractional area of a 20X field. Typically, dilutions that give 30–50 cells per field are chosen for quantification. Comparison of viral titers performed by LFU to that obtained by PFU assays reveals identical results when infections are performed as described in Section 3.3. prior to agar overlaying.

An alternatively useful method of titering viral stocks by FFU is based on similar principles as described above with the exception of detecting either transgene expression or viral protein expression using immunofluorescent techniques.

1. Subconfluent monolayers of 293 cells grown in 24-well tissue culture dishes are infected with serial dilution of viral stocks made in DMEM/1%PS (ranging from \(10^{-4}\)–\(10^{-9}\)).
2. 100 µL of each dilution is placed in each well for 2 h.
3. Add 10 µL FCS to each well and incubate for an additional 18 h.
4. Following infection, the media is aspirated and cell monolayers are fixed in –20°C methanol for 10 min followed by air drying.
5. Methanol-fixed cells are blocked in 20% goat serum/PBS (0.45-µm filtered) for 30 min followed by incubation in a 1:10 dilution of FITC-labeled goat anti-Ad3 antibody in 1.5%GS/PBS for 90 min.
6. Cells are washed three times in 1.5%GS/PBS and covered with 100 μL Citifluor antifadent (University of Kent at Canterbury, England).
7. Fluorescent cells are visualized using an inverted fluorescent microscope and quantitated as described in Section 3.3., step 1 for the LFU assay (see Note 4).

3.4. In Vivo Delivery of Recombinant Viruses to the Lung

Multiple model systems have been used to study recombinant adenoviral gene delivery to the lung, including transtracheal instillation, selective bronchoscopy of lung subsegments followed by instillation of virus, and nasal inhalation. We will review the most widely used method of transtracheal administration as applied to small rodent animal models such as the mouse and cotton rat.

1. 80–120 g cotton rats are anesthetized with isoflurane.
2. The neck is cleaned with an isopropyl alcohol swab and a 1-cm midline incision is made with a sharp scalpel.
3. The trachea is exposed by blunt dissection and a small, 3-mm incision is made through the muscle surrounding the trachea.
4. 1–5 × 10¹⁰ infectious particles (as measured by PFU, LFU, or FFU) in a total volume of 100–150 μL in PBS is instilled through a tuberculin syringe followed by 300 μL of air.
5. The incision is closed with two staples and the animals are returned to their cages.

Similar procedures can be performed on mice by reducing the total dose and volume of virus according to the total weight (kg) of the animal. Typically a 25-g mouse tolerates a dose of 2 × 10⁹ PFU of recombinant virus instilled in a total volume of 25–35 μL.

3.5. Harvesting of Lungs for Histologic Analysis

The lungs from animals infected with E1-deleted recombinant lacZ adenovirus typically express highest levels of transgene activity between 3 and 7 d. Several methods of tissue preparation have been employed in an attempt to analyze multiple experimental endpoints, including histopathology, using formalin inflation fixed lungs; localization of lacZ transgene activity, by in situ histochemical staining of lungs by inflation; and multiple histologic endpoints such as immunocytochemistry, histochemistry, and in situ hybridization, in lungs inflated with OCT for frozen sectioning. In this review we will summarize the most flexible of these techniques used for frozen sectioning.

1. Animals are euthanized by an overdose of 200 mg/kg pentobarbital.
2. The thorax is opened and the proximal end of the trachea is exposed and canulated with an 18-gage angiocath 3-mL syringe filled with 37°C 1:1 mixture of OCT embedding medium in PBS. We have adopted this method of in situ inflation to avoid accidental nicking of the lungs upon removal, which will result in insufficient inflation pressures.
3. Gentle pressure is applied until the lungs are visually inflated within the thorax. Typically, inflation of a cotton rat lung requires 2 mL of OCT.
4. Once inflated, the heart-lung cassette is removed and quickly cooled in ice-cold PBS for 10 min.
5. The hardened lung cassette is removed and trimmed of nonlung tissue on an ice-cooled surface.
6. The lung is cut into large (approx 1-cm) wedges and placed in plastic embedding blocks filled with 100% OCT embedding medium such that the cut face is in contact with the bottom of the block.
7. Blocks are frozen in a pulverized dry ice/isopentane slurry. Since some air may be retained within the lung tissue, care should be taken to manipulate tissue samples during freezing to retain placement on the bottom of the block.
8. 6-μm frozen sections are cut using a cryostat and processed for multiple histologic analyses such as immunocytochemistry, histochemistry, and in situ hybridization.

Similar methods of lung inflation can be used to retain better morphology, which is useful in histopathologic evaluation by inflation with buffered formalin followed by embedding in paraffin for sectioning. Alternatively, lungs can also be stained in situ with X-gal by inflation fixation in 0.5% glutaraldehyde/PBS for 10 min, followed by two sequential 15-min lavages with 1 mM MgCl2/PBS and staining in X-gal staining solution for 1 h at 37°C. Following staining in X-gal, lungs should be washed with PBS twice by lavage, instilled with buffered formalin/1% glutaraldehyde, and postfixed overnight before processing in paraffin for sectioning.

3.6. Histochemical Detection of LacZ Transgene Activity
1. 6-μm frozen sections are fixed in 0.5% glutaraldehyde in PBS for 10 min. Care should be taken to avoid repeated freeze thawing of freshly cut sections before processing for histochemical staining. Hence, slides are precooled and sections only thawed once for placement onto slides. Once sections are placed on slides they should be kept within the cryostat (−18°C) until staining and not air dried. Alternatively, slides can be stored at −80°C for several weeks without substantial loss of β-galactosidase activity.
2. Sections are washed twice for 15 min with 1 mM MgCl2/PBS, placed in X-gal staining solution (solution 4) for 4–6 h, followed by washing two times in PBS and postfixed in buffered formalin containing 1% glutaraldehyde for 15 min (see Note 5).
3. Sections are counterstained briefly in hematoxylin (without blueing). Alternatively, sections stained in neutral red also provide good contrast of blue X-gal precipitate.

3.7. Immunocytochemical Detection of LacZ Transgene Activity
1. Frozen sections (6-μm) are postfixed in −20°C methanol for 10 min followed by air drying.
2. Sections are blocked with (0.45-μm filtered) 20% goat-serum in PBS for 30 min.
3. Blocking solution is replaced with 60 μg/mL of rabbit anti-β-galactosidase (5′→3′) in the presence of 1.5% goat serum/PBS for 90 min.

4. Sections are washed three times for 8 min in 1.5% goat serum/PBS.

5. Sections are incubated with 5 μg/mL of a goat antirabbit-FITC antibody in 1.5% goat serum/PBS for 30 min.

6. Sections are washed three times for 8 min in 1.5% goat serum/PBS.

7. Coverslip sections in antifade (Citifluor) and visualize by fluorescent microscopy.

3.8. In Situ Hybridization Detection of Transgene-Expressed mRNA

In situ hybridization of transgene-expressed mRNA has proven to be an extremely powerful tool in the assessment of gene transfer efficiencies from non-β-gal recombinant vectors. Because analysis of therapeutic transgene expression is most often assessed within a setting of endogenous gene expression, we have developed two approaches to circumvent this problem: the use of probe sequences from the transgene that are not conserved across species, and transgene-specific probes based on unique sequences inserted in the 3' untranslated region of the expressed transgene mRNA. These two methods have been extensively used in several animal models analyzing recombinant adenoviral expression of CFTR in the lung (2,4).

1. Frozen sections (6-μm) are mounted on positively charged slides (Probe-On Plus, Fisher, Pittsburgh, PA) and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 3 h. Care should be taken to keep sections at -18°C while sectioning and to fix sections as soon as possible.

2. Following fixation, sections are dehydrated through graded concentrations of ethanol consisting of mixtures of depC-treated water and ethanol at 30, 50, 70, 95, and 100%. After this fixation step, care should be taken to use RNase-free precautions for all of the subsequent steps (see Note 6).

3. Sections are then desiccated overnight under vacuum and stored at -20°C until in situ analysis is performed.

4. Before prehybridization, sections are treated with 10 μg/mL proteinase-K (solution 6) for 30 min at 30°C, rinsed twice in 0.2X SSC for 30 s each and fixed in 4% paraformaldehyde/PBS for 20 min.

5. Following fixation to inactivate proteinase-K, sections are rinsed twice for 4 min in 0.1M triethanolamine [TEA] (pH 8.0), incubated in 0.25% acetic anhydride/0.1M TEA (pH 8.0) for 10 min at room temperature (RT), rinsed in 0.2X SSC twice for 2 min, and followed by dehydration in graded concentrations of ethanol as described in Section 3.8., step 2. Sections are again dried under vacuum for 3 h to overnight.

6. Sections are submerged in prehybridization buffer (solution 8) for 4 h at 54°C. Alternatively, RNase control sections are treated with 200 μg/mL RNase A pre-treatment buffer (solution 7) for 1 h at 37°C followed by fixation in 4% paraformaldehyde/PBS for 20 min to inactivate RNase. Following fixation, slides are
Adenovirus Transfer to Airway Epithelium

rinsed twice for 2 min in 0.2X SSC before the prehybridization step (RNase-pretreated slides serve as a negative control for mRNA specific hybridization).

7. Slides are removed from the prehybridization solution and coverslipped with 25 μL of hybridization buffer (solution 9) containing 1 × 10⁷ cpm/mL ³⁵S antisense or sense probes. The edge of each coverslip is then sealed with DPX mounting media. Slides are incubated flat in a 54°C oven for 16–18 h. Probes were synthesized with the Promega (Madison, WI) in vitro transcription system using 0.25 mCi of ³⁵S UTP and ³⁵S CTP as the sole source of these nucleotides for transcription from the T7 and SP6 RNA polymerase promoters. A detailed method of in vitro transcription reaction is outlined in Section 3.9

8. Following hybridization, slides are washed in 4X SSC for 20 min at room temperature (four 5-min changes), treated with RNase (20 μg/mL) posthybridization wash buffer (solution 10) for 30 min at 37°C, rinsed in 2X SSC/1 mM DTT at room temperature for 20 min (four 5-min changes), and finally washed for three 15-min changes of 0.5X SSC/1 mM DTT at 54°C.

9. Slides are dehydrated through graded concentrations of ethanol (30% ethanol/0.1X SSC/1 mM DTT; 70% ethanol/0.1X SSC/1 mM DTT, 95% ethanol, 2X 100% ethanol) and air dried prior to dipping in undiluted photoemulsion (Kodak, Rochester, NY).

10. Slides were developed at 1 and 2 wk and analyzed by bright- and dark-field microscopy.

3.9. Generation of In Situ-Radiolabeled Probes

The generation of radiolabeled RNA probes with high specific activity is critical to the success of sensitive in situ mRNA detection protocols. To this end, we have developed a technique utilizing PCR-generated template DNA for in vitro transcription reactions. Using this methodology, highly specific probes can be generated with 50 ng of template DNA. Additionally, this methodology allows for the flexible generation of multiple probes to different portions of the cDNA without the need for subcloning into riboprobe plasmids. Ideally, PCR templates should be 500–600 bp in length. This probe length allows for the greatest sensitivity without encountering barriers of probe penetration. Typically, we will generate our PCR riboprobe templates by using cDNA template primers in which SP6 and T7 RNA polymerase binding sites have been added to the 5' end of the oligonucleotide (Fig. 1). Additionally, we have incorporated a notI cloning site into our primers for use in generating probes for which plasmid templates are not readily available and genomic DNA is used as template. These additional sequences also assure adequate secondary structure of PCR fragment DNA for RNA polymerase binding by avoiding terminal DNA binding sites that may be less favorable for transcription initiation. The PCR-amplified template DNA is purified by low melting point agarose gel electrophoresis followed by organic extraction in 2X phenol, 1X phenol:CHCl₃ once, and 2X CHCl₃, followed by ethanol precipitation.
Fig. 1. The PCR primer sequences for generation of template DNA for in situ riboprobes. Additional sequences homologous to the cDNA of interest are added to the 3'-end of primer sequences shown. Typically, these cDNA homologous sequences are 24 bp long with a 50% GC content. Such primers will have a melting temperature of 72°C and provide minimal background in PCR reactions with annealing temperatures of 68°C.

1. 0.25 mCi of both S\textsuperscript{35}CTP and S\textsuperscript{35}UTP are combined into a 0.5-mL Eppendorf tube, frozen on dry ice, and lyophilized within a Speed-vac.
2. To the radioactive pellets, the following components of the Promega in vitro transcription kit are added: 4 µL of 5X buffer, 2 µL 100 mM DTT, 1 µL of ATP, 1 µL of GTP, 1 µL of template DNA (50–100 ng of PCR template), 0.5 µL RNasin, 9.5 µL depC H\textsubscript{2}O, and 1 µL of T7 or SP6 RNA polymerase.
3. Reactions are incubated for 1 h at 37°C, followed by the addition of 0.5 µL RNasin, 1 µL RQ1 DNase, and incubation for 15 min at 37°C.
4. 80 µL of depC H\textsubscript{2}O are added and the resultant mixture extracted with 100 µL of phenol:chloroform (1:1) and chloroform, followed by the addition of 25 µg E. coli tRNA and precipitation twice with ammonium acetate (final concentration 0.7M) and 2.5 vol of −20°C ethanol to remove unincorporated nucleotides.
5. Probe pellets are resuspended in 10 mM Tris-HCl, pH 8.0, 10 mM DDT, 1 mM ETDA, 0.3% SDS, and frozen on dry ice in 10-µL aliquots. Probes can be stored at −134°C for 1–2 wk without substantial increases in background. However, best results are usually obtained with fresh probe. The specific activity of probes generated by this methodology is generally 1–2 × 10\textsuperscript{7} cpm/ng template DNA.

4. Notes
1. We have found that increased yields of recombinant adenovirus are obtained from infection with purified stocks rather than crude lysates owing to nonspecific cytopathic effects caused by cell debris within the crude lysates.
2. The time necessary to achieve cytopathic effect (CPE) varies for different recombinant vectors. Hence, plates should be monitored closely following infection within the 30–48 h window to determine the optimal time for harvesting based on
Acienovirus Transfer to Airway Epithelium

1. Generation of CPE. Appropriate harvesting based on optimal CPE will affect yields in the production of virus.

3. It is important to note that the length of viral infection will significantly affect the accuracy of LFU and FFU titering if infections are longer than 21 h. This results from the amplification of viral replication within 293 cells and artifactual elevation of the apparent titer.

4. In all fluorescent antibody assays, the addition of antifade is critical to avoid photobleaching that will make quantification and photography difficult.

5. Postfixation in formalin/1% glutaraldehyde helps to minimize leaching of X-gal precipitate in organics used for dehydration of tissue sections before coverslipping in xylene-soluble permount.

6. Care should be taken to avoid RNase contamination during the in situ hybridization procedure. To this end, all solutions are made with depC-treated water and dedicated reagents. Most reagents cannot be treated directly with depC. Hence all solutions should be made in depC-treated glassware or RNase-free plasticware with depC-treated autoclaved H2O. When it is necessary to pH an RNase free solution, this should be performed by aliquoting a small amount into a secondary vessel to avoid direct contact of contaminated electrodes with in situ solutions. Gloves should be worn at all times. In addition, all glassware is baked at 250°C for 20 h and plasticware is treated by submerging in water containing depC for 16-20 h, followed by autoclaving to inactivate depC.

Acknowledgments

I gratefully acknowledge the support and advice of James Wilson in the preparation of this review and the research that has gone into the development of these technologies. Much of the technology cited in this review is the result of research supported by the NIDDK grant ROI DK47967 (J.F.E.).

References


Methods for Retrovirus-Mediated Gene Transfer to Fetal Lung

Bruce R. Pitt, Margaret A. Schwarz, and Richard D. Bland

1. Introduction

With the advent of improved vectors for DNA delivery, somatic gene therapeutic approaches have expanded rapidly in the last few years. The vast majority of applications include ex vivo and in vivo protocols in patients postnatally. Nonetheless there is increasing interest and compelling reasons to consider prenatal application of somatic gene therapy (1,2). In the current chapter, we will review theoretical, ethical, and experimental support for in utero gene therapy and then outline the methodology and large animal model we are currently using to consider retrovirus-mediated gene transfer to fetal lung. In this latter regard, the candidate inherited disorder is cystic fibrosis and the reader is referred to Chapters 1 and 12 in this volume.

1.1. Human Fetal Gene Therapy

The potential for human fetal gene therapy coincides with advances in molecular medicine and recent developments in fetal medicine including advances in prenatal human genetics, ultrasonography and surgical, anesthetic, and tocolytic techniques (3). The most compelling reason to consider fetal gene therapy is that the disorder may result in irreversible and life threatening morbidity in the postnatal period (including adolescence and maturity) that may be prevented by corrective gene therapy in utero, at a time when pathophysiology of the disorder is minimal. Advantages for fetal gene therapy relate to our current understanding of available DNA delivery systems and thus the immunologically primitive fetus with rapidly dividing and proliferating tissues may provide a more permissive environment for gene-transfer protocols. For ethical considerations, fetal gene therapy is limited to somatic and not germ cell
transfer (4,5). Thus, in spite of enormous technical advances in embryonic stem cell manipulations and/or homologous recombination, ethical and logistic issues suggest that genetic manipulation of early embryonic human stem cells is not likely to be a part of fetal gene transfer in the foreseeable future.

1.2. Gene Delivery to Extrapulmonary Tissue in Postimplantation Embryonic Experimental Animals

Original observations on fetal gene transfer involved the use of retroviruses (see also Chapters 1,9,15,17,20,27 in this volume). Jaenisch first reported widespread expression of Moloney murine leukemia virus (M-MuLV) specific sequences in mouse tissue after injection of replication-competent virus into 8–9 d gestational age (g.a.) mice (6). Subsequently, Jaenisch and colleagues (7) used a replication-defective retrovirus to express human c-ha-ras-1 oncogene in mouse tissue after direct microinjection of viral supernatant into the 8.5 d g.a. conceptus. Others have reported expression of transgenes in the central nervous system of fetal rat pups after plasmid (8) and replication-defective retroviral (9) DNA transfer.

Many of the current preclinical approaches to rational fetal gene therapy relate to transduction of fetal hematopoietic tissue. Many laboratories have been able to transfect bone marrow cells in culture. Concerns about longevity of expression, problems with obtaining and transducing totipotent stem cells, and potential requirements for bone marrow ablation to facilitate the graft, however, have prompted alternative strategies. Depending on gestational age, fetal blood, and/or liver are important sources of hematopoietic precursors, and at this stage, potential stem cells proliferate more rapidly to facilitate fetal bone marrow development. Kantoff et al. (10) obtained fetal blood cells from 93–105 d g.a. sheep (term: 147 d), infected them ex vivo with a replication-defective M-MuLV-based vector containing cDNA to neomycin-resistance gene, and returned the transduced fetal hematopoietic cells to the lamb via an indwelling catheter. Neomycin-resistance gene sequences were detected in progenitor cells for up to 2 yr. Alternatively, Clapp et al. (11) directly injected replication-defective retrovirus into fetal liver of rat pups (11–18 d g.a.; term: 22 d) and noted transgene expression and proviral integration in bone marrow, thymus, and spleen for 6–7 mo postnatally. More recently, transduced cells obtained from human cord blood have been used in a clinical trial for treatment of adenosine deaminase deficiency in neonates (12).

In a parallel vein, gene transfer to mature liver, in experimental animals (13) and patients (14), has been accomplished by infection of cultured hepatocytes followed by hepatocellular transplantation of transduced cells into the portal vein. A similar ex vivo approach for hepatocellular transplantation in utero has recently been described (15,16) and the potential for enhanced graft survival in
the immunologically incompetent fetal liver seems favorable. Direct in vivo retrovirus-mediated gene transfer to liver has also been accomplished (17) in partially hepatectomized mature rats and after ip injection in utero in rat pups (14 d g.a.).

1.3. Gene Transfer to Fetal Lung

Gene therapy to mature lung has advanced to the stage of several important clinical trials for cystic fibrosis (18). Although the target tissue for complementation with normal cystic fibrosis transmembrane conductance regulator (CFTR) gene remains somewhat obscure, the respiratory epithelium appears to be one useful site. The peculiarities of the anatomy of lung preclude ex vivo approaches, and, accordingly, somatic gene transfer to lung has been vigorously pursued (19,20). Adenoviral (18) and cationic liposomal (21) vectors have been employed, as they may transfer DNA to nonreplicating cells that are representative of the normally quiescent respiratory epithelium. Intrinsic to the current nature of these vectors is that expression of transgene is transient, and in the case of adenoviral vectors, potential local inflammatory responses are possible. Several investigators have noted potential advantages to somatic fetal gene therapy for CF (and other pulmonary disorders) including:

1. Potential limited immune response of the fetus, thereby minimizing inflammation and perhaps prolonging adenovirus-mediated transgene expression (22);
2. Increased number of accessible dividing cells (and perhaps stem cells), thereby allowing use of retroviral vectors (23);
3. Greater expression of CFTR in fetal than adult lungs suggesting an important, but yet undetermined, role for fetal alveolar CFTR (24,25);
4. Interestingly, in light of point 3, above, the impression that fetal lungs of patients with CF are structurally normal (26); and
5. Minimal numbers of macrophages and protein as well as less complex surface lining material than the mature lung, thereby facilitating delivery of macromolecules including DNA in the fluid-filled future airspaces.

Some principles of this approach that may support human applications have been outlined in experiments in vitro (27,28). Currently, the most efficient vector for somatic lung gene transfer, e.g., adenovirus, has shown some promise after introduction into amniotic fluid of fetal sheep and mice (29) and rats (30). It is, however, associated with surprisingly significant inflammatory response when directly instilled in fetal sheep airways (22,31).

In addition to CF, other diseases that are candidates for fetal gene therapy include inherited disorders such as alpha-1 antitrypsin deficiency and surfactant apoprotein-B deficiency as well as chronic lung injury of early infancy, an iatrogenic disorder often called bronchopulmonary dysplasia.
This chapter outlines our initial approaches with a large animal model of retrovirus-mediated gene transfer to fetal sheep lung. As noted, reports from other investigators (22,31) have outlined a similar approach using adenoviral vectors.

2. Materials

2.1. Retroviral Supernatant Production

We used MFG retroviral vectors in which LacZ or secreted (human interleukin receptor antagonist protein or growth hormone) cDNAs were inserted, as recently described (32–34). The MFG vector is a simplified retroviral vector derived from M-MuLV in which the polymerase and envelope gene sequences are deleted to render the virus replication defective. Partial gag sequences were retained to increase packaging efficiency of the unspliced transcript. The inserted cDNAs were transcribed from promoter/enhancer sequences from the retroviral long terminal repeat. High titer amphotrophic producer of recombinant retrovirus was obtained by cotransfection of plasmid (pSV2neo) into packaging (CRIP) cells NIH3T3 cells were cultivated in DMEM and supplemented with glucose (4.5 g/L) and 10% v/v heat inactivated calf or sheep serum, penicillin (100 U/mL), and glutamine (200 mg/mL), and were infected with the virus. Conditioned medium containing viral supernatants were tested for their titer and presence of helper virus, concentrated 3X, and stored at −70°C.

2.2. Fetal Surgery

Twin fetal lambs (104–117 d g.a.) were surgically prepared with chronic catheters as previously described (31,32). The ewe was sedated with ketamine (15 mg/kg, im) and then underwent a hysterotomy with halothane and nitrous oxide anesthesia. The uterus was opened with a small incision and polyvinyl catheters were placed directly into the fetal carotid artery, jugular vein, and trachea. A fluid-filled catheter was also placed in the amniotic sac. Catheters were sutured to the ewe’s uterus and abdomen. Wounds were closed with silk sutures and catheters were exteriorized and placed in a pouch sewn on the ewe’s flank. Antibiotics were injected into the amniotic sac (penicillin [1,000,000 U] and kanamycin [400 mg]) and fetal vein (penicillin [300,000 U] and gentamicin [10 mg]) at the time of surgery and daily thereafter. The ewes also received 5 mL of a mixture of penicillin and dihydrostreptomycin (Combiotic, 200,000 U/mL procaine of penicillin G and 250 mg/mL dihydrostreptomycin, Pfizer, New York) and 600 mg kanamycin, im each day. Hematocrit, arterial pH, and blood gas tension were measured every other day.
3. Methods

3.1. Administration of Retrovirus to Fetal Lambs

1. 1–2 d after surgery, blood was withdrawn from the carotid artery catheter for determination of hematocrit, pH, and blood gas tension.
2. We then withdrew 30–60 mL of tracheal fluid into a sterile syringe.
3. A 10-mL solution of MFG-viral supernatant (3 × 10^6 particles/mL) was brought to room temperature immediately before use and polybrene (see Note 4) was added to a final concentration of 2 μg/mL. Viral supernatant was delivered directly into the tracheal catheter (over 1–2 min) and was flushed into the lungs with 30–60 mL of previously withdrawn lung liquid.
4. The identical procedure was performed on the other twin with the other MFG-vector.
5. All catheters were flushed with heparinized saline and antibiotics were given.
6. The same procedure was repeated on three consecutive days.

3.2. Histochemical Techniques

1. The ewe and her fetuses were killed with an overdose of sodium pentobarbital (50 mg/kg, iv). In some cases, the fetuses were aborted spontaneously after 1–3 wk.
2. Lungs were removed and inflated, ex vivo, to 30 cm H₂O transpulmonary pressure by injecting a phosphate-buffered solution containing 4% paraformaldehyde and 0.1% glutaraldehyde (pH 7.4) into the trachea. Lungs were immersed in PBS containing 30% sucrose and stored at 4°C.
3. Small blocks of tissue were obtained from proximal and distal sections of each lobe and snap frozen in liquid nitrogen. Frozen sections were cut on a Reichart-Jung 2800E Cryostat (Cambridge Instruments, Boston, MA) and mounted on poly-L-lysine-coated glass slides.
4. For histochemical localization of β-galactosidase activity, small blocks of fixed tissue were rinsed in PBS and incubated in a solution containing 1 mg/mL 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal; Sigma, St. Louis, MO) in Tris phosphate-buffered saline (pH 8.0) with 5 mM potassium ferricyanide and potassium ferrocyanide.
5. Tissue was incubated for 4–24 h (optimal time was 8 h) and washed in PBS.
6. Tissue was then frozen and sectioned as in step 3. Sections were counterstained with eosin and hematoxylin and observed with a light microscope.
7. Alternatively, standard immunocytochemical techniques can be performed on the same fixed tissue (see steps 2 and 3).

4. Notes

1. We have performed experiments outlined above using MFG-based replication-defective retroviral vectors to express β-galactosidase and human interleukin receptor antagonist protein in the lungs of developing sheep (37). We have noted detectable levels of β-galactosidase via histochemistry in the upper and lower airways of some of these animals as long as 3 wk after infection. Localization of β-galactosidase was most prominent in epithelial cells of proximal airways with
additional histochemical appearance associated with fibroblasts and macrophages in the submucosa. In addition, relevant developments from other laboratories (22,30,31) as well as new and novel retroviral constructs (38) highlight some of the concerns and potential future directions of in utero retrovirus-mediated gene transfer to lung. The approach may be most applicable to inherited disorders such as cystic fibrosis or surfactant apoprotein B deficiency, in which a potential lifetime therapy may ultimately be exacted after a limited therapeutic application in utero. Obviously, such a theoretical goal requires lifetime expression of the therapeutic gene with minimal toxicity to the patient (both fetus and mother). Thus, issues of efficiency, longevity of expression (including retroviral shutdown), progenitor cell expression, and potential germ cell contamination will have to be considered along with developments in fetal surgery and in utero genetic diagnosis to bring these issues to clinical consideration.

2. Sheep are the animal of choice for long-term in utero physiological studies. The uterine muscle is thinner and does not contract as vigorously as the primate, thereby minimizing surgery-induced abortion. Since the sheep is polycotyledenous, there is less likelihood of separating the placenta during surgery (36). The fetus is relatively large (3–5 kg at term) and facilitates intrapulmonary maneuvers as well as greatly simplifying chronic catheterization and sample withdrawal. A significant portion of lung development occurs prenatally, and thus a large portion of the replicating cells is likely to be accessible, especially during early gestation (39). Unlike primates, twin pregnancies are common in sheep, thus providing a useful control. Unfortunately, sheep are less than ideal with respect to serving as a model for human disease. There are no known spontaneous cystic fibrosis lesions in sheep, nor is there the likelihood that transgenic knockout models will be created for this disorder in this expensive, large, mammal that takes approx 5 mo to develop in utero. In this regard, progress with in utero mouse surgery and micromanipulation will be of great value.

3. We have focused on applications of retroviral vector-mediated gene transfer to fetal lung. Alternative vectors may (or may not) be as useful. For instance, adenoviral vectors, although extraordinarily efficient in mature, nondividing airway cells, are intrinsically transient, and current generation vectors have produced significant intrapulmonary inflammatory response in the fetus (22,31). Adeno-associated viral vectors also work well in nondividing cells and appear ideal for gene transfer for CF in mature lung (40). Their application for in utero transfer has not been reported as of this date although they appear to be useful candidates (41). In our initial experience (37), we instilled almost 10^8 viral particles over 3 d to produce a modest level of expression that lasted at least 3 wk in the fetal lung. Limitations of overall pattern and level of expression may have included:
   a. The absolute number of cells undergoing mitosis at the time of infection;
   b. Number of cells with appropriate surface receptors for the amphotropic receptor required to recognize recombinant MFG-based vectors,
   c. In situ shutdown of retroviral transgene, and
   d. Retroviral titer.
Retroviral Transfer to Fetal Lung

It is possible that infection of an earlier age fetus may be useful since the number of proliferating cells is likely to be greater, and cell types with different sensitivities to retroviral infection may be apparent (42). Alterations in the envelope protein may also be useful since it has been shown that switching from a murine-based vector to Gibbons Ape Leukemia virus backbone enhanced gene transfer to respiratory epithelium in vitro (43). Reports of extraordinarily high titer pseudotyped retrovirus are also likely to enhance efficiency of in utero retrovirus-mediated gene transfer (38).

4. Several problems occurred in our initial experiments. We noted significant pulmonary toxicity that led to early fetal demise in most of the animals studied. The toxicity was most likely owing to the presence of polybrene, a cation used to enhance viral entry into cells, that may have been pneumotoxic (44). Initial attempts at using a secreted reporter gene (e.g., human IRAP) for prospective assessment of gene transfer revealed that expression was not sufficient to readily detect measurable levels in either fetal or maternal plasma or amniotic fluid. Furthermore, the presence of transgenic protein in the retroviral supernatant made determinations 1 wk after infection ambiguous.

We used a relatively invasive surgical procedure to introduce viral supernatant. Advances in fetal medicine and surgery make the likelihood of more acceptable procedures such as video fetoscopy, potentially useful for gene therapy. Issues of germ-line transmission remain speculative.

Acknowledgments

This work was supported in part by the Cystic Fibrosis Foundation (BRP), NIH HL32154 (BRP), and NIH HL49098 (RDB). MAS is a clinician-scientist of the American Heart Association.

References


In Situ Retrovirus-Mediated Gene Transfer into the Liver

Nicolas Ferry, Sophie Branchereau, Jean-Michel Heard, and Olivier Danos

1. Introduction

Gene transfer into hepatocytes is a promising approach for the treatment of genetic liver diseases. Candidate diseases for human trials are life-threatening disorders resulting from a single genetic defect that do not compromise other liver functions and the organization of the hepatic tissue. Orthotopic liver transplantation has been successfully performed in patients with genetic liver disease, but their use is limited by the high mortality risk, the need for chronic immunosuppression, and the availability of organs. Gene therapy would provide an alternative to transplantation by restoring the expression of the defective gene in an organ that is otherwise structurally and functionally normal.

Hepatocytes can be isolated from a liver biopsy, grown in vitro, genetically modified with retroviral vectors, and reimplanted by injection into the portal vein or in the spleen. This ex vivo approach has been successfully used in animal models and in human patients (7–9,12,20), but the in vitro step is highly labor intensive and introduces contamination risks.

Alternative techniques have taken advantage of the fenestrated nature of the vascular endothelium in the liver, that allows direct contact between the hepatocytes and substances carried by the blood stream. A simple portal injection of recombinant retroviruses (2,13,14) or adenoviruses (11,16) results in the uptake and expression of the foreign genetic material by the hepatocytes. A more thorough perfusion of the whole organ, which requires a temporary isolation from the circulation, increases gene transfer efficiency (3,5,18).

In this chapter, we describe the procedures that we have developed in mice, rats, and dogs for retrovirus-mediated gene transfer into hepatocytes in vivo.
by selective perfusion of the regenerating liver (2,5,3). The technique includes a partial hepatectomy that triggers hepatocyte division, making them susceptible to retroviral infection. Liver perfusion is performed at the time of maximal mitotic activity in the regenerating liver. The organ is isolated from the circulation by clamping efferent and afferent vessels, and perfused for 10–15 min with a retroviral vector preparation. The asanguinous perfusion ensures a complete and blood-free irrigation of the parenchyme with the retroviral vector, without spreading to other tissues.

The proportion of dividing cells at a given time after hepatectomy varies between species. In rats, where this has been thoroughly studied, the peak of mitotic activity is observed around 24 h, with around 15% dividing hepatocytes at this time (4). This sets the upper limit of expected gene transfer efficiency with the current retroviral vectors. Using an amphotropic retrovirus vector encoding an nls-lac Z reporter gene, we routinely obtain around 1% of hepatocytes expressing the nucleus-targeted β-galactosidase for periods of time longer than 1 yr (2,5). These procedures can be performed by investigators with basic surgical skills. Following the recommendations detailed here, a survival rate above 90% is expected.

2. Materials

2.1. Production of Recombinant Retroviruses

1. Retroviral vector supernatant is prepared according to procedures described in Chapter 3. We use supernatant from YCRIP or YCRE packaging cell lines freshly harvested or from a stock frozen at −80°C.
2. Polybrene: 0.8 mg/mL solution in PBS (100X stock solution, stable at 4°C).
3. Cellulose acetate 0.45-μm sterile filters
4. Concentration can be obtained by ultrafiltration on Amicon cells or Filtron macrosep tubes. For large volumes (>100 mL), tangential filtration on a Sartorius device is used (Sartocon Mini SM 17521 with a Polysulfon membrane, porosity 100,000)

2.2. Surgery

1. Sterile surgical tools are required to perform hepatectomy and liver perfusion. Microsurgery tools are used for mice and rats
2. Retractors adapted to rats and mice.
3. Ackland clamp C1
4. Mosquito forceps: curved, 4.5 cm.
5. Bulldog clamps: straight and curved, 4.5 cm.
7. Scissors: straight, heavy, and blunt, blades 12 cm.
8. Scissors: straight, 10 cm.
11 Iris forceps: delicate, straight, 1-mm jaws
12. Dissection forceps: curved and straight with 12-cm teeth
13 Microvascular forceps 3 and 5.
15. Needle holder: O'Brien, curved, extra delicate with 0 8-mm jaws
16. Silicone irrigator with irrigating canula olive tip.
17. Electrocoagulator.
18. Sterile cheese cloths and sterile physiologic serum.
19. 4/0 vicryl suture, 8/0 monobrin suture, 4/0 and 2/0 ethircin suture, 10/0 silk suture.
20 Intraflon 22-gage catheter.
22. Silastic tubing 0.012 in. id x 0.025 in. od
23. Peristaltic pump with connecting tubing (Masterflex digistaltic pump, Cole Parmer, Niles, IL)
24. Surgical microscope

3. Methods

3.1. Production of Recombinant Retroviruses

Amphotropic retroviral vector is produced according to usual procedures and harvested from the supernatant of confluent packaging cells. Optimal conditions for virus production including cell density, type of support, media, and temperature may vary from one producer to the other (15).

Small-scale production of under 100 mL of viral supernatant is usually sufficient for experiments in rats and mice. Liver perfusion in large mammals requires the production of more than 500 mL, which has to be conducted in roller bottles or in large-scale tissue culture devices such as Nunc's "cell factories." Production of large batches is also required before concentration. The best results are obtained with freshly concentrated virus. However, it is convenient to use aliquots of a calibrated concentrated stock kept at −80°C. A procedure for 10–30-fold concentration of large batches by tangential flow filtration has been described (17). If needed, small volumes can also be concentrated 10-fold using 300-kDa cutoff membranes on an Amicon ultrafiltration cell (2–3 h at 4°C) or a Macrosep centrifugal concentrator (3500g for 1 h).

3.2. Anesthesia

3.2.1. Rats

Rats are readily anesthetized using ether. Anesthesia is induced by placing animals in a tightly covered glass recipient containing a piece of surgical cotton presoaked in ether. After placing the animal on the surgical table, anesthesia is maintained by securing the body of a 50 mL-syringe containing an ether-wetted piece of cotton in front of its nose.
3.2.2. Mice

Ether is not recommended in mice and we use intraperitoneal injections of ketamine or Avertin at 150 mg/kg. Pentobarbital, which is mainly metabolized by the liver, induces very long anesthesia in partially hepatectomized mice.

3.2.3. Dogs

General anesthesia is induced with pentobarbital (20 mg/kg of body wt) and maintained with 0.5% fluothane administered through endotracheal tube. Antibiotic therapy (amoxicillin, 100 mg/kg/d) is given preoperatively and for 3 d postoperatively. Feeding is stopped 12 h before surgery and restarted progressively after 24 h.

3.3. Partial Hepatectomy

In rats and mice, two-thirds partial hepatectomy is essentially carried out as described by Higgins and Anderson (10)

3.3.1. Rats

1. After opening the abdominal cavity by midline incision, the liver is exposed by retracting the abdominal walls with small retractors. The liver ligaments are then carefully sectioned with fine scissors. Care must be taken at this step to avoid any injury of the liver pedicule vessels.
2. The bases of the median and left lateral lobe is then surrounded with a 4/0 vicryl suture. After controlling the correct position of the suture, it is tightened carefully and secured by two or three knots. Both lobes should rapidly turn dark red, indicating that the blood flow has been stopped. They are then sectioned and the stump is replaced in the abdominal cavity.
3. The right adrenal vein is usually sutured at the time of partial hepatectomy. For doing this, the caudate lobe is retracted to expose the right adrenal pedicule. The right adrenal vein is then carefully dissected and sutured with a 4/0 vicryl suture. The abdominal wall is closed in two steps (muscle and skin) using a 2/0 ethircrin suture with a straight triangular needle.

3.3.2. Mice

The procedure is essentially the same as for rats. However, better results are obtained when the two large lobes are removed successively. The gallbladder has to be left intact. The hepatectomy in mice is more conveniently carried out under a surgical microscope. Care must be taken to avoid compression of the hepatic pedicule by suture knots. This would compromise the inward blood flow through the remnant liver lobes. The right adrenal vein is usually electrocoagulated rather than sutured. The abdominal wall is closed as for rats, using a 4/0 ethircrin suture.
3.3.3. Dogs

1. The far left lobes, corresponding to 44% of the liver mass, are removed (6).
2. The abdomen is opened via a long midline incision and the falciform and the left triangular ligaments are divided.
3. The hilar structure (bile ducts, hepatic arteries, and portal veins) is ligated first and the liver transection is performed by carefully crushing the tissue with a Kelly forceps (Kelly clamp).
4. The two hepatic veins are suture-ligated.
5. The abdominal wall is closed without drains. The resected lobes must be weighed to estimate the remnant liver mass.

3.4. Asanguineous Perfusion

After a lag period allowing the regenerating liver to reach the time of maximal mitotic activity (24 h in mice and rats; 72 h in dogs) (see Note 1), animals are again anesthetized and the abdominal cavity is opened by removing the sutures placed at the time of hepatectomy.

3.4.1. Rats

1. The abdominal cavity is largely exposed using two, or preferably four, retractors. The intestinal mass is moved to the left side of the animal and maintained in a sterile cheese cloth, wetted with physiologic serum. It is very important to check that the intestine remains wetted during the whole surgical procedure.
2. The portal vein and the hepatic artery are localized and carefully dissected. The gastroduodenal vein is also dissected at its junction with the portal vein. Two sutures (4/0 vicryl) are placed on the gastroduodenal vein, which is then sectioned between them to free the portal vein from any attachment.
3. The infrahepatic vena cava is also dissected in its upper renal portion. The suprahepatic vena cava is carefully freed from any adherence. This can be difficult if the hepatectomy stump is too large and hides this portion of the vena cava. After all dissections are completed, the liver is ready to be isolated from the blood circulation.
4. For this, an angulated microclamp is placed on the hepatic artery and bulldog clamps are successively placed on the portal vein, the infrahepatic vena cava, and the suprahepatic vena cava under the diaphragm. Under microscopic viewing, the wall of the portal vein is carefully opened, and a silastic tube is introduced into the opening. This tube is connected to a larger tube placed in a peristaltic pump that ensures a constant flow through the liver. A 22-gage catheter is placed in the infrahepatic vena cava and connected to a tube in order to direct the flow to a waste recipient placed below the level of the animal. The perfusion is started by turning on the peristaltic pump, with an inward flow of 4–5 mL/min.
5. At the end of the perfusion, the portal vein is carefully sutured under the microscope using 10/0 silk suture. Another clamp is placed on the infrahepatic vena cava just upstream of the 22G catheter to isolate the opening of the vena cava.
The portal, hepatic artery, and suprahepatic clamps are then released to restore a normal portal flow. This leaves a few minutes to suture the infrahepatic vena cava using a 10/0 silk suture.

6. It is essential that the clamping of the portal vein in rats does not exceed 20 min, to avoid irreversible ischemic lesion of the intestine.

3.4.2. Mice

In mice, the protocol is essentially the same except that:

1. We usually place only one suture with no section of the gastroduodenal vein. The hepatic artery is electrocoagulated to avoid traumatic injury of the hepatic pedicle during dissection.
2. The portal vein and infrahepatic vena cava are clamped with Ackland clamps.
3. The perfusion usually requires lower volumes of retrovirus-containing medium and can therefore be performed using a hand-controlled syringe instead of a peristaltic pump.
4. After clamping the vena cava, we frequently observe a respiratory arrest in mice. This requires a mechanical ventilation system during the clamping period. A simple tube connected to a bulb, in which the nose of the animal is placed, is sufficient to ensure ventilation. The frequency and volume of air can be directly monitored by the observation of the amplitude of the diaphragm movements at each insufflation.
5. The outward catheter placed in the infrahepatic vena cava is smaller in mice (30G). Because of rapid clotting, the suture of the vena cava is not necessary if the opening of the vein wall has been kept small. It is sufficient to apply a piece of cheese cloth to ensure correct clotting. The whole procedure is usually carried out in less than 10 min.

3.4.3. Dogs

In dogs, the perfusion is performed 72 h after partial hepatectomy.

1. The inferior vena cava is mobilized above and below the liver to allow the placement of the vascular clamps.
2. The right adrenal vein is ligated to avoid bleeding during the perfusion, since the clamp cannot be applied above its insertion. The blood inflow is occluded by placing a vascular clamp on the hepatic pedicle (in mass or selectively on the portal vein and the hepatic artery). The vena cava is then clamped, first below and then above the liver (I).
3. The portal vein is canulated with a 14-gage catheter (Vialon, Becton Dickinson, Rutherford, NJ).
4. A suction cannula used for collecting the perfusion outflow is placed into the infrahepatic vena cava through a 4-mm incision of the anterior wall, made above the clamp.
5. The single pass perfusion is started via the portal catheter at 1 mL/min/g of estimated remnant liver. The flow rate can be moderately and transiently increased...
to ensure a satisfactory perfusion of all segments. The average duration of the perfusion is under 20 min. The liver is then rinsed with PBS and the perfusion is stopped.

6. The portal vein and the vena cava incisions are then sutured with a 5/0 nylon monofilament, and the liver is revascularized by declamping first the portal vein, then the vena cava below, and finally, above the liver.

7. The abdominal wall is closed in two layers, without drainage.

3.5. Analysis

3.5.1. Histology

In order to prepare histological sections on which the activity of the introduced gene can be revealed, the liver is fixed in situ by perfusion.

1. The anesthetized animal is placed on the surgical table and the abdominal wall is opened. The portal vein is carefully dissected and a butterfly 27-gage needle is secured into the vein. The thorax is opened and the lower vena cava is sectioned. Immediately afterward, the liver is first rinsed by perfusing physiologic serum (20–40 mL in rats; 5–10 mL in mice; 200 mL in dogs).

2. After washing, the organ is perfused with a 4% paraformaldehyde solution freshly prepared in PBS.

3. Once the liver has hardened, it can be easily removed from the animal and minced with a razor blade in 5-mm thick fragments. These fragments are rinsed in PBS and immersed overnight at 4°C in a 30% sucrose solution freshly prepared in PBS.

4. The fragments are then ready to be snap-frozen in dry ice or liquid nitrogen-refrigerated isopentane, and cryostat sections can be prepared.

3.5.2. DNA Analysis

Detection of the transgene after retroviral mediated transfer can be performed by PCR or Southern blot analysis on total liver DNA.

1. Liver fragments are immediately frozen by immersion in liquid nitrogen. The tissue is then finely powdered in liquid nitrogen.

2. The powder is incubated for 3 h at 50°C in 5 mL of a mixture containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 1% SDS, and 500 μg/mL proteinase K.

3. At the end of the incubation, 5 mL of phenol is added and mixed gently by rotational agitation for 1 h. After centrifugation (2000g for 2 min), the aqueous phase is collected and phenol is extracted once.

4. The high-mol-wt DNA is then extracted twice for 10 min with chloroform: isoamylalcohol (24/1, v/v).

5. DNA is then precipitated with 1 vol of isopropanol and the high-mol-wt DNA precipitate is collected using the tip of a Pasteur pipet and dissolved in 500 μL of sodium acetate (300 mM). DNA is then precipitated again with 2 vol of ethanol at −20°C for 30–60 min and centrifuged for 30 min. The pellet is washed with 1 mL of 70% ethanol, centrifuged, and air dried.
6. The final pellet is then resuspended in 0.5–1 mL of water and stored at 4°C until used. PCR is run on 0.5–1 μg of high-mol-wt DNA using two synthetic oligonucleotides as primers, one of which is complementary to the vector sequence and the other one to the transgene (5). We usually performed 40 cycles of amplification at 95°C for denaturation, 55°C for hybridization, and 72°C for elongation under conditions recommended by the Taq polymerase supplier. The PCR assay allows the detection of 1 positive cell in 10^4, and is therefore not much more sensitive than direct staining for a histochemical marker or *in situ* hybridization (7).

4. Notes

1. Retroviral vectors derived from murine leukemia viruses will integrate in dividing cells only (19). Therefore the perfusion procedure should be carried out at the time of maximal hepatocytes division (2). In rats and mice, the peak of DNA synthesis in hepatocytes occurs 24–30 h following partial hepatectomy. In dogs, the regeneration process is somewhat slower and the perfusion is carried out 3 d after partial hepatectomy. When working on a specific animal model, one has to first delineate the kinetics of liver regeneration to ensure an optimal gene transfer by perfusing during the peak of DNA synthesis.

2. Different animal strains may react differently to our surgical procedure. Examples are renal ischemia after vena cava clamping in Fischer rats or the exquisite sensitivity to hepatectomy resulting in high level of mortality in the C3H mice strain. We recommend, whenever possible, to use Wistar or Lewis rat strains or C57Bl6 mice to carry out liver perfusions.

3. Simplified methods for retrovirus-mediated gene transfer into the regenerating liver have been described. In these procedures, there is no complete vascular exclusion of the liver at the time of infection, and the viral supernatants are either slowly injected into the portal stream (13) or bolus-injected in the portal vein after single clamping of the portal vein (2,18). In our hands, the complete vascular exclusion procedure results in a 10-fold increase in gene transfer efficiency when compared to portal bolus injection technique (2). This may be owing in part to the smaller volumes of vector-containing supernatant that can be infused during a single bolus injection. In addition, the organ is not washed free of blood during the bolus injection. This may decrease efficiency since retroviral particles can bind to blood cells still in the liver sinusoids or, depending on the animal species, be inactivated by the complement.

4. The titer of the viral supernatant perfused into the liver is a critical parameter. We have observed that most viral particles originally present in the perfusion fluid were able to escape the liver. This was evidenced by a minimal (<10%) decrease in the viral titer measured in the outflow. Better results are always obtained with freshly collected, high titer viral preparations. A titer of 10^5 viral particles/mL (assayed on NIH 3T3 cells) is the minimum required to achieve easily detectable gene transfer into the liver of rodents.
Transfer into the Liver

References


Methods for Delivery of Genes to Hepatocytes In Vivo Using Recombinant Adenovirus Vectors

Darlene Barr and Mark A. Kay

1. Introduction

1.1. The Liver as a Target Organ

In many ways, the liver represents an ideal target organ for gene delivery. Anatomically, the sheer bulk of its tissue mass and its dual blood supply are advantageous for intravascular injection of virus into either portal or systemic circulation. The portal vein provides a direct iv route into the liver. It also theoretically provides an indirect route by oral administration since the portal system drains the gut.

The liver is the site of production of a large number of proteins functioning in a wide variety of physiologic functions including metabolism, coagulation, cholesterol packaging, detoxification, and other enzymatic activities. Hepatic deficiencies result in human diseases that are potentially correctable by introduction of the cDNA or gene expressing the missing protein. Examples of these diseases include inborn errors of metabolism, such as tyrosinemia (type 1), phenylketonuria, ornithine transcarbamylase deficiency, and alpha 1-antitrypsin deficiency. Other hepatic protein deficiencies include lipoprotein receptor deficiencies that result in considerable cardiovascular mortality. In theory, exogenous proteins can be produced since hepatocytes are well equipped for secretion into plasma. Erythropoietin is one example of a protein that may be heterotopically produced in the liver with potential benefit to thousands with anemia secondary to chronic renal failure.

1.2. Gene Transfer into Hepatocytes

A number of different vectors and procedures have been developed for hepatic gene transfer. Both ex vivo and in vivo methods have been used. These
approaches have been reviewed (1,2). The widely used retroviral vectors require dividing hepatocytes for gene transfer, resulting in low transduction rates (1–2%), but long-term gene expression.

1.3. Advantages of Adenoviral Vectors for Hepatocyte Transduction

Adenoviruses are linear, double-stranded DNA viruses that are unencapsulated and icosahedral. They are pathogenic in humans but infection generally results in a mild upper respiratory disease. Most adults have been exposed to a type of adenovirus. Of the more than 43 serotypes identified in humans, serotypes 2 and 5 are the most widely used as vectors. None have been associated with malignancy in humans and their safety has been demonstrated by use of live viral vaccines in the 1970s (3,4). In addition to its safety, adenovirus (Ad) is an attractive vector from the viewpoint of the molecular biologist. The Ad life cycle has been extensively studied and its genome mapped (3). The Ad genome is divided into early (E1–E4) and late (L1–L5) transcription regions. The virus can be rendered replication defective by deleting the E1 region. After E1 deletion, the virus can still be grown and manipulated in vitro in a permissive cell line, such as 293, Ad 5-transformed human embryonic kidney cells that supply E1 in trans. Additional deletions in the E3 region can be utilized to accommodate a fairly large amount of foreign DNA, up to 8.5 kb (3,5,6). Advanced generation vectors deleted for various portions of the viral genome are being developed by a number of different laboratories. Adenovirus is easily concentrated and purified to titers in the range of $10^{11}$–$10^{12}$ viral plaque forming units (PFU)/mL.

The adenovirus exhibits a natural tropism for the respiratory tract but is also capable of transducing many other tissues, including the liver. A major advantage of using adenovirus is the ability to transduce nondividing cells in direct contrast to retroviruses. Efficient transduction of hepatocytes has been demonstrated both in culture and in mouse models. Approximately 95–100% of hepatocytes can be transduced in vivo by injecting $1 \times 10^{10}$ viral PFU systemically (7). Recombinant adenovirus has been used for hepatic gene transfer to express therapeutic gene products in mice (8–10), rats (11), rabbits (12), and dogs (13).

1.4. Disadvantages of Adenoviral Vectors

Despite the advantage of high levels of transgene protein production, the expression is transient, disappearing over a period of weeks to months. The mechanism for shutdown is related to cell-mediated immunity against infected cells (14). Furthermore, secondary transduction of hepatocytes using adenoviral vectors has been unsuccessful, likely owing to the generation of neutralizing antibodies after the first infusion (9,15).
In this chapter, we describe the details of intraportal and systemic iv methods of delivering adenoviral vectors in a mouse model. The principles are the same in other animal models as briefly described at the end.

2. Materials

1. Dialysis Buffer: 10 mM Tris HCl pH 8.0, 1 mM MgCl₂, 10% glycerol
2. Human 293 cells grown in Ham's DMEM with 10% fetal calf serum, and 1 mM glutamine, penicillin/streptomycin is optional.
3. Mice. 12–30 g.
4. Ear tags or ear punch device for animal identification.
5. Avertin (tribromoethanol). Stock Solution. Mix 25 g of 2,2,2-tribromoethanol in 25 mL tert-amy1 alcohol. Store at room temperature. Protect from light. Keeps for 2 mo. Working solution: Mix 1.2 mL stock solution in 98.8 mL PBS (12 mg/mL). Store at 4°C in foil wrapped bottle. Keeps 2–3 wk
6. Buprenorphine
7. Mouse restraining device
8. For portal vein injection: cork board; cotton tipped swabs. Surgical instruments: iris scissors, Adson forceps with teeth, nontoothed forceps, needle holder, 3-O Vicryl or silk suture on a tapered needle, Gelfoam (absorbable gelatin sponge, Upjohn, Kalamazoo, MI) in 0.5 × 0.5-cm squares. Warming lights (40-watt incandescent desk lamp), tuberculin or insulin syringes with 27- or 30-gage needles, and microcentrifuge.

3. Methods

Instructions for preparation of the adenovirus for infusion into mice will be detailed. These techniques may be modified for use in other large and small animal models. General instruction in the care and handling of rodents is available in ref. 16. Mice should be housed in accordance with standards of the institutional animal care board. Decontaminate equipment with 5% SDS or bleach prior to autoclaving.

The viral stock can be diluted in PBS or basal media without serum. Generally, we dilute virus to 0.1 mL per injection dose. Methods for both intraportal and iv injection via lateral tail veins will be described. Generally, most of the virus is taken up by the liver by either injection method (17). We inject about $1 \times 10^{10}$ PFU per animal to achieve close to 100% hepatocyte gene transduction. After portal vein infusion, up to 50 copies of adenoviral genomes are present per diploid host DNA (7,17). Lesser amounts of adenovirus can be infused for lower rates of gene transfer (7,17). Portal vein injection requires a laparotomy. Both techniques are performed with a general anesthetic. We suggest intraperitoneal Avertin (tribromoethanol). Tail vein injections can be performed without an anesthetic when one becomes facile with the technique.
3.1. Adenovirus Preparation

1. The methods used are modified from Graham and Prevec (4).
2. Grow a human cell line such as 293 to 70% confluence. Remove media from cells.
3. Add the thawed adenovirus at a multiplicity of infection (MOI) of 10 viral. Incubate at room temperature for 1 h, occasionally swirling the plate.
4. Replace with 10 mL fresh media per 10-cm dish. Incubate until full cytopathic effect is noted, usually 36–48 h later.
5. Harvest cells by pipeting media until they lift off the plate and centrifuge gently to pellet cells. Add small volume of PBS. Total volume about 36 mL/125–10-cm dish or 50–15-cm dish.
6. Lyse cells. Thaw cells/PBS at 37°C. Repeat freeze and thaw two more times.
7. Spin out cellular debris on a table-top centrifuge before layering lysate on step gradient.
8. Step gradient: Form gradient by carefully layering CsCl solutions of the following densities in an ultraclear SW 40 Ti tube: 6 mL cell layer, 3 mL 1.25 g/cm³, 3 mL 1.35 g/cm³, 0.5 mL 1.5 g/cm³.
   Spin for 1 h at 35,000 rpm, 12°C, acceleration profile 1, deceleration profile 1.
9. Adenovirus will appear as a bluish-white band at the interface between the 1.25 and 1.35 g/cm³ layers. Collect virus. Spin virus in a second continuous 1.35 g/mL CsCl gradient. Spin in an SW 40 Ti rotor at 35,000 rpm for 16 h. Remove adenovirus (blue white band).
10. Dialyze virus overnight (50,000 mol wt cutoff) in dialysis buffer.
11. Quantitate Ad viral DNA at OD₂₆₀, 25-µL aliquot in 475 µL of 0.1% SDS, 10 mM Tris. One OD unit equals about 10¹² particles/mL. Generally, the particle titer is 20–100-fold higher than the number of plaque-forming units. Plaque titer can be performed as described in ref. 4.
12. Store in aliquots at −80°C.

3.2. Preparation

For survival experiments, mark the animal in some manner. Tail marking will be removed with normal grooming in 2–3 d. For long-term experiments, numbered ear clips work well. Patterns of triangular and circular ear punches can also be used, but the pattern must be clearly identifiable and checked periodically for distortion from chewing or healing over.

3.3. Anesthetic Management

1. Grasp the mouse tail at the base with the dominant hand. Allow the mouse to catch an edge of the cage with his forefeet. Firmly grasp posterior aspect of neck and shoulders with the nondominant hand to prevent head mobility and biting. Cradle the animal in the palm and turn abdominal side forward. Turn the animal head down to displace abdominal viscera from the lower abdomen. Inject 0.5–1.0 mL Avertin in the right lower quadrant (0.5 mL for a small 10–20 g mouse and 0.8–1.0 mL for larger mice). This dose provides approx 30 min of anesthesia of suitable depth for laparotomy.
2. Gage the depth of anesthesia by first observing for motion and breathing. The animal should be motionless except for steady respiratory effort. Second, test the response to pain by pinch or pin prick to the web space of the foot. If withdrawal of the limb is noted, an abdominal incision will not be tolerated and 25% of the dose should be repeated. If no respirations are noted and the eyes are white and glassy, the mouse is overanesthetized.

3.3. Laparotomy (for Portal Vein Injection)

1. Place the mouse supine on a cork surgical board or other flat surface. Secure extremities with tape or pins in the web spaces of feet.
2. Shave the abdomen in the midline using a small electric shaver or with light scraping using a scalpel. Swab with skin antiseptic.
3. Make a vertical midline incision using iris scissors. Tent up the skin of the abdominal wall fascia by grasping just lateral to midline with an Adson-toothed forceps to prevent injury to bowel.
4. With two fingers, gently rotate the intestines in the right lower quadrant to the left exposing the superior mesenteric vein. (The portal vein is formed by the confluence of splenic and superior mesenteric veins.) The bowels will stay in this position if covered by a moistened gauze.
5. Retract the liver lobes cephalad with a cotton tipped swab. The superior mesenteric and portal veins should be clearly visible. Confirm the identity of the portal vein by tracing its entry into the hepatic hilum.
6. Retract the small bowel mesentery inferolaterally with the nondominant hand to hold the portal vein taut.
7. Inject the viral preparation in the portal vein (as close to the confluence of splenic and superior mesenteric veins as possible) with the dominant hand. The total volume of the infusion should not exceed 0.5 mL (we generally use 0.1 mL) and should be room temperature.
8. Withdraw the needle carefully and place a small piece of Gelfoam over the puncture site. Gelfoam, combined with gentle digital pressure, should result in hemostasis in 3–5 min. Too much pressure will cut off portal flow and will injure the liver if held for long periods of time.
9. Lift the Gelfoam off slowly with a forceps to check hemostasis. If the Gelfoam does not peel easily, add a drop or two of saline to soak it off. If still bleeding, reapply Gelfoam and pressure. If hemostasis is satisfactory but bleeding recurs after several attempts to remove the Gelfoam, it can be left in place; there is, however, an increased risk of infection.
10. Replace the intestines and liver lobes to their anatomic positions.
11. Close the skin and abdominal wall en bloc with a continuous suture of 3-0 silk or Vicryl. Warm saline placed in the peritoneal cavity prior to closure will help prevent dehydration.
12. Recover the animal under warming lights and examine every 15–20 min until ambulating. Provide free access to food and water. When fully recovered, animals can be observed grooming, ambulating, and feeding. If the animal exhibits signs of pain, i.e., listlessness, administer butenorphine 2.0 mg/kg sc every 12 h.
3.4. Tail Vein Injection

1. Anesthetize mice as described.
2. Place prone on a flat surface.
3. Warm mouse tails under a lamp to induce vasodilation.
4. Lay the tail flat on the table and stroke with the thumb for further venodilation.
5. Identify the paired lateral tail veins on both sides of midline of the dorsum of the tail. They are dark blue structures that may be better appreciated in lower levels of light and by rotating the tail from side to side.
6. Steady the tail proximally with the index finger and distally with the thumb (nondominant hand).
7. With the dominant hand, insert the needle into the vein at a 45° angle from the horizontal plane of the table. (A slight bend in the needle is sometimes helpful, but take care not to lacerate the vein.) Start at the midportion of the tail so that one may move more proximally if the first attempt fails.
8. Inject with slow gentle pressure. If the needle is correctly positioned in the lumen of the vein, the injection will go in easily. If any force is required, the needle has become displaced and the injection will be sc if one is able to inject at all. (With too much force, the syringe will pop off, splashing virus in your face.) Occasionally one may observe a successful injection flowing downstream by noting the color change from the dilute infusate.
9. Withdraw the needle and apply firm digital pressure until hemostasis is assured. Recover the mice as described in Section 3.3., step 12. No postoperative analgesics should be required.

3.5. Large Animal Models

For larger animals, the scale-up in concentration of virus is roughly proportional to their size. For transduction of rat hepatocytes, we infuse $1 \times 10^{11}$ PFU in 100 g animals. In dogs weighing 12–17 kg, we have perfused the portal vein with about $1.5 \times 10^{11}$ PFU/kg and have obtained more than 1 adenovirus genome/copy per diploid copy of host DNA (13). Kozarsky and Wilson used $1.5 \times 10^{15}$ viral particles (about $1.5 \times 10^{11}$ PFU) in 2–4 kg rabbits to achieve approx 100% hepatocyte transduction and $4 \times 10^{12}$ particles to achieve 50–75% transduction (12).

4. Notes

Most of the adenovirus is taken up by the liver by the injection routes described; however, a significant amount is taken up to lesser degrees by other tissues. (See Kay et al. [13], Smith et al. [9], and Vrancken-Peeters et al. [17] for tissue distribution.) The relative distribution of adenovirus is the same if the virus is administered by peripheral or portal vein infusion. More recently, a method of transducing hepatocytes by infusion via the biliary tract has been described in the rat (18) and mouse (14). The method leads to similar hepatocyte transduction. Additionally, biliary epithelial cells are transduced at greater
frequencies than by the vascular infusion methods. The persistence of gene expression varies among different mouse strains (15).

5. Summary

Adenoviral vectors provide an efficient vehicle for hepatic gene delivery. Their use has been complicated by the immunologic response directed against transduced hepatocytes and the inability to retransduce cells because of humoral immunity. It is of interest that between different inbred mouse strains there can be a wide variation in the persistence of gene expression (15). There is cautious optimism that less antigenic vectors (19–21), possibly in combination with immunomodulatory agents (22), may allow for improved adenovirus-mediated gene transfer in animals and ultimately in humans.

References


Methods for Producing High-Titer, Pantropic Retroviral Vectors for Gene Transfer into Leukemic T-Cells

Jane C. Burns, JING-KUAN YEE, and Alice L. Yu

1. Introduction

Current limitations to the use of Moloney murine leukemia virus (MoMLV)-derived retroviral vectors as a tool for gene transfer include the inability to obtain high-titer vector stocks and the narrow host cell range of these vectors. To overcome these disadvantages, we developed a new class of pantropic retroviral vector that has a broadened host cell range and can be concentrated to very high titers (>10^9 colony forming units [CFU]/mL) (1).

1.1. Pantropic Retroviral Vectors

Pseudotyped pantropic vectors contain the vesicular stomatitis virus G glycoprotein (VSV-G) substituted for the retroviral envelope protein (Fig. 1). The host range of the amphotropic and ecotropic retroviral vectors is limited to cells that express a specific protein receptor on the cell surface that acts as a ligand for the envelope protein. The human amphotropic virus receptor encoded on chromosome 8 has recently been cloned and sequenced (2). The VSV-G envelope protein, in contrast, binds to phospholipid components in the cell membrane, thus permitting these VSV-G pseudotyped particles to enter virtually every cell with a plasma membrane (3). The extremely broad host cell range of these vectors has been demonstrated by infection of a wide variety of organisms including cows, goats, fish, newts, and insects (4,5 and JCB, unpublished observations).

An additional consequence of the substitution of VSV-G for the retroviral envelope protein is increased stability of the virus particle. Pantropic vectors...
can be concentrated by simple physical methods such as ultracentrifugation to high titer (10^8–10^9 CFU/mL) with greater than 75% recovery of the total number of infectious particles (I). This method allows generation of vector stocks at titers sufficient for micoinjection of nanoliter volumes into small cavities. Such approaches have permitted the creation of transgenic fish by micoinjection of concentrated vector into the developing fish embryo (5). Future human gene therapy applications may include direct injection into tumors or into discrete vascular beds for which small volume, high titer vector stocks are essential.

1.2. Application of Pantropic Vectors to Gene Therapy of Leukemia

The goal of gene therapy in leukemia is, simply stated, to convert cells with the malignant phenotype into normal cells. This can most easily be conceptualized as a treatment of cells in the bone marrow that would reconstitute normal gene function and restore the normal phenotype. To make such an approach successful, the following conditions should be met:

1. Identification of a discrete gene or genes whose mutation has resulted in the altered phenotype or whose reconstitution will result in a normal phenotype.
2. Availability of a method for therapeutic gene delivery that will allow every malignant cell to be treated.

Retroviral vector treatment could be administered either in vivo by direct iv injection of retroviral vectors or ex vivo by treatment of residual leukemic cells harvested from the bone marrow followed by reinfusion of these treated cells into the host.

Acute leukemia in childhood is an appropriate target for this type of therapeutic approach. Bone marrow transplantation (BMT) is considered the treatment of choice for acute myeloblastic leukemia (AML) in first remission and for acute lymphoblastic leukemia (ALL) in second remission. Since the majority (75%) of BMT candidates do not have an HLA-matched sibling, autologous BMT provides an important alternative, if the residual leukemic cells in the marrow can be eliminated. Current strategies to immunologically purge the bone marrow of residual leukemic cells often fail to eliminate all malignant cells. Gene therapy strategies to restore normal tumor suppressor gene function (e.g., p53) in the leukemic cells may be a useful adjunct to eliminate residual malignant cells. The easy accessibility of leukemic cells for preclinical studies make leukemia an ideal model for gene therapy. In addition, evidence is now mounting for the presence of cancer cells in the bone marrow and circulation in a variety of other malignancies, such as breast cancer, lung cancer, and neuroblastoma. Thus, gene therapy treatment of the bone marrow may have broader application to a number of solid tumors as well as hematologic malignancies.

We describe here a method to generate helper virus-free, high titer pantropic vector stocks for infection of primary human leukemic T-cells. This approach has been successfully used to infect leukemic cells harvested from the bone marrow and peripheral blood of pediatric patients with newly diagnosed or relapsed T-cell acute lymphoblastic leukemia (T-ALL). The pantropic vector was more efficient than the corresponding amphotropic retroviral vector with the identical genome in infecting human leukemic T-cells.

2. Materials

2.1. Cell Lines and Media

1. PA317 mouse fibroblasts (6), 293 gag-pol human kidney epithelial cells (1), and 208F rat fibroblasts (7) are grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with 4.5 gm/L glucose and supplemented with 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (2 mM).
2. Human T-cells are grown in RPMI-1640 supplemented with 10% fetal calf serum, antibiotics, and L-glutamine as in item 1.
3. Syringe-tip filters (0.45- and 0.2-µm).
2.2. Reagents and Solutions for Cell Culture, Infection of T-Cells, and Concentration of Pantropic Vector

1. Polybrene stock solution (4 mg/mL): Dissolve 0.4 g of hexadimethrine bromide in 100 mL of sterile water and store at 4°C.
2. Protamine sulfate stock solution (2.5 mg/mL): dissolve 250 mg protamine sulfate (tissue culture grade, Sigma, St. Louis, MO) in 100 mL PBS. Filter sterilize and store at 4°C.
3. G418 stock solution (40 mg/mL): Dissolve 4 g active Geneticin (Gibco-BRL, Gaithersburgh, MD) in 100 mL 0.1M HEPES, pH 7.9, filter sterilize, and store at 4°C.
4. Phosphate-buffered saline (PBS). 137 mM NaCl, 2.6 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄. Autoclave and store at room temperature.
5. 0.1X Hank's Balanced Salt Solution (HBSS): Dilute 1 mL HBSS in 9 mL sterile ddH₂O and store at 4°C.
6. Auszyme EIA kit, Abbot Laboratories, North Chicago, IL
7. Giemsa stain.
8. Phorbol 12-myristate 13-acetate (PMA) stock solution: Reconstitute the contents of the vial containing PMA with DMSO to make a final concentration of 20 μg/mL. Store at -20°C.
9. Calcium ionophore stock solution: Dissolve 5.2 mg in DMSO to give a final concentration of 2 mM. Store at -20°C.
10. rIL-2 stock solution: 1 x 10⁵ U/mL in RPMI. Store at 4°C.

2.3. Reagents for Detection of Retroviral Genome in Infected Cells

1. Cell lysis buffer: 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 25 mM EDTA, 0.5% SDS, and 200 μg/mL proteinase K. Store buffer at 4°C and add proteinase K (frozen stock) just before use
2. 10X Tag buffer: 500 mM KCl, 100 mM Tris-HCl, pH 8.4, 15 mM MgCl₂. Store at 4°C.
3. PCR primers for S gene: Dilute to a final concentration of 625 μM. Use 1 μL each primer/reaction.
   a. Upstream primer sequence for S gene: 5' GGC TAT CGC TGG ATG TGT CT 3'.
   b. Downstream primer sequence for S gene: 5' ACT GAA CAA ATG GCA CTA GT 3'.
4. 10X dNTP stock solution: dilute dCTP, dUTP, dATP, and dGTP with ddH₂O to a final concentration of 2 mM. Store at 4°C.

3. Methods

3.1. Production of Pantropic Vector

Pantropic vectors can be produced with a wide variety of genes and internal promoters. Described here is the production of the pantropic vector LSRNL (8) in which the Hepatitis B surface antigen gene (S) is expressed from the 5'
Fig. 2. Genetic organization of vector, LSRNL. LTR or L, Moloney murine leukemia virus long terminal repeat; HBsAg or S, Hepatitis B surface antigen; RSV or R, Rous sarcoma virus long terminal repeat; neo or N, neomycin phosphotransferase.

Moloney murine leukemia virus LTR (L) and the neomycin phosphotransferase gene (N) is expressed from the Rous sarcoma virus LTR (R) (Fig. 2). This retroviral construct has the advantage that clones may be screened for S antigen production by simply sampling the culture media from each clone and using the Auszyme EIA kit to easily and quickly determine the relative amounts of S antigen in the supernatant. This process identifies the clone that is most likely to produce high titers of pantropic vector when the cells are subsequently transfected with the VSV-G plasmid.

The general method for production of pantropic virus is illustrated in Fig. 3. In the first step, amphotropic virus is produced in PA317 cells. In the second step, this virus is used to infect 293 cells stably expressing the MoMLV gag and pol genes. Pantropic virus particles are produced when the 293 gag-pol/LSRNL cells are transfected with a plasmid expressing the VSV-G protein under the control of a strong promoter (hCMV).

All work with pantropic retroviral vectors is performed according to Biosafety Level-2 (BSL-2) practices. It should be remembered that, although pantropic vectors are replication incompetent, they readily infect a broad range of cells including all dividing human cells. Care must be taken to avoid creating aerosols of the virus except inside a tissue culture hood equipped with a HEPA filter.

3.7.7. Generation of Stable 293 gag-pol Producer Cell Line

1. Day 1: Seed approx 1–5 × 10⁵ PA317 cells in a 10-cm tissue culture plate.
2. Day 2: Transfect by standard calcium phosphate coprecipitation (9) with 20 µg pLSRNL.
3. Day 3: Seed approx 1 × 10⁶ 293 gag-pol cells in a 10-cm tissue culture plate for infection on d 4.
4. Day 4: Aspirate and filter (0.45-µm) supernatant from PA317 cells transfected on d 2. Use 1–2 mL to infect 293 gag-pol cells in the presence of 8 µg/mL polybrene.
5. Day 5: At least 16 h after infection, aspirate culture medium from infected 293 gag-pol cells and replace with fresh medium containing 400 µg/mL of G418. Continue to refeed culture with fresh medium containing G418 until resistant colonies appear in 12–14 d. Because 293 gag-pol cells adhere only loosely to the
Fig. 3. Method for production of pantropic retroviral vectors.

tissue culture plate, care must be taken to avoid disturbing the colonies when changing the medium

6. Week 2: Aspirate all medium from the plate of colonies. Pick individual G418-resistant colonies by placing 3 µL of media directly over colony with a micropipetor. Aspirate up and down to dislodge the cells and transfer to G418-containing medium in a 24-well plate. Repeat the procedure until 10–20 colonies have been selected.

7. To screen colonies for S gene production, aspirate 200 µL of culture media per clone (8). Use the manufacturer’s instructions for Procedure B to test supernatants for the amount of S antigen secreted into the medium.

3.1.2. Generation of Pantropic Vector

1. Day 1: To generate pantropic vector, choose the clone with the highest production of S antigen and seed a 10-cm plate with 1–5 × 10⁵ cells.

2. Day 2: Transfect these cells by calcium phosphate coprecipitation with 20 µg of the plasmid hCMV-VSV-G. Remove the calcium phosphate-DNA precipitate
after 6–8 h and gently replace medium. Care must be taken not to disturb the monolayer, as the cells are fragile and poorly adherent after transfection. Longer incubation with the precipitate will kill the cells.

3. Day 3: Seed a 10-cm plate with $1 \times 10^5$ 208F rat fibroblasts for titering virus.

4. Days 4–6: Collect virus-containing culture supernatant once a day beginning 48 h after transfection. The highest titer virus is produced at 48–96 h post-transfection. Culture supernatant should be filtered (0.45-μm) and an aliquot removed to determine virus titer. Remaining supernatant may be frozen at −70°C until needed.

5. To titer virus, dilute appropriately with DMEM (e.g., anticipate $1 \times 10^6$ cfu/mL, use 20 μL of 1:1000 dilution of virus per 10-cm plate) and add diluted virus to 208F cells in the presence of polybrene (8 μg/mL).

6. After overnight incubation with virus and polybrene, replace medium with DMEM complete medium containing G418 (400 μg/mL). Replace medium every 3–4 d until colonies are visible (usually 10–14 d).

7. Fix colonies in methanol and stain with Giemsa stain to facilitate counting colonies. Calculate titer of virus. Assume that each colony represents one infectious unit of virus.

### 3.2. Concentration of Pantropic Vector

1. UV sterilize swinging buckets, caps, and ultracentrifuge tubes for an SW41 or other suitable swinging bucket rotor.

2. Under sterile conditions, transfer viral culture supernatant to the centrifuge tubes, and tighten caps.

3. To pellet virus particles, centrifuge at 50,000g (25K in an SW41 rotor) for 90 min at 4°C. Decant supernatant and wipe inside walls of tube with a tissue (nonsterile Kimwipe works well) to remove excess medium. Place tube upright in ice and add 30 μL 0.1X HBSS/tube. Allow invisible pellet to resuspend overnight at 4°C.

4. Resuspend pellet by pipeting vigorously up and down but avoid foaming the solution. Concentrated vectors stocks may be frozen at −70°C for at least 3 mo without loss of titer (see Note 3).

### 3.3. Infection of T-Cells

1. Separate leukemic cells from bone marrow by Ficoll-Hypaque centrifugation.

2. Pre-incubate mononuclear cells with PMA (20 ng/mL) and 2 μM calcium ionophore for 3 h at 37°C.

3. Suspend cells in RPMI-1640 supplemented with 10% fetal calf serum and 200 U/mL IL-2.

4. Aliquot $1 \times 10^7$ cells/tube and incubate overnight at 37°C with 5 μg/mL protamine sulfate and virus stock (MOI 0.5–2.0) added to culture medium (see Note 2).

5. Wash cells and resuspend in RPMI with additives. Analyze at 48 h after infection for presence of provirus in cells (see Note 1).
3.4. Detection of Provirus in Infected T-Cells

A polymerase chain reaction (PCR) assay can be used to detect provirus in infected T-cells. A positive PCR signal documents that attachment, uncoating, and reverse transcription of the RNA genome into the DNA provirus have successfully occurred. It should be noted that the following assay detects infected cells containing proviral DNA but does not provide information about integration of the provirus into the host cell genome (see Note 4).

1. Lyse approx 1 x 10^5 cells infected 48–72 h previously with the pantropic vector, LSRNL, in 500 μL cell lysis buffer at 37°C overnight.
2. Isolate the DNA following phenol extraction and isopropanol precipitation and resuspend in 25–100 μL sterile ddH₂O.
3. Quantitate the concentration of DNA spectrophotometrically and use 0.5–1 μg DNA per PCR reaction.
4. Add the DNA to a 500-μL Eppendorf tube containing 50 μL of mineral oil. Centrifuge briefly and heat at 100°C for 2 min. Quickly chill on ice.
5. Prepare the PCR reaction mix (25 μL final volume) by mixing 0.5 μM each primer, 200 μM dNTP’s, 2 U Taq DNA polymerase, 2.5 μL 10X Taq buffer, and sufficient water to equal 25 μL when mixed with the DNA. Chill reaction mix on ice and add to DNA by centrifuging briefly so that reaction components are below mineral oil overlay.
6. Place the reaction tube in a thermal cycling device and proceed with the following thermal profile: 94°C for 3 min, followed by 40 cycles of 94°C for 1 min, 58°C for 40 s, and 72°C for 40 s. End with a final extension step at 72°C for 5 min.
7. Analyze 10 μL of PCR reaction mixture on a 2% agarose gel stained with ethidium bromide. If additional sensitivity is required, the resolved DNA can be transferred to a nylon membrane and hybridized with a labeled S PCR product probe to visualize the predicted 334 bp amplification product.

4. Notes

1. In addition to the virus particles with their RNA genome, culture supernatants also contain contaminating S protein as well as DNA fragments from the producer cells containing provirus. It is not possible, therefore, to reliably measure S antigen in the T-cell culture supernatants with the Auszyme kit, as one cannot distinguish between preformed S antigen from the 293 gag-pol/LSRNL cells and newly synthesized S antigen from the LSRNL-infected T-cells. Similarly, cells must be washed extensively to remove contaminating provirus derived from the 293 gag-pol/LSRNL cells before DNA is extracted for PCR analysis to confirm T-cell infection. Alternatively, a method of DNA extraction in which nuclei are separated from membranes before lysis may be used.
2. Protamine sulfate must be used as the polycation during infection of T-cells as polybrene is toxic to primary T-cells, even at low concentrations (>4 μg/mL). Poly-l-lysine (5 μg/mL) may also be used.
3. With one round of centrifugation, vector stocks can easily be concentrated 100-fold with >90% recovery of infectious particles (1). If further concentration of vector is required, a second round of centrifugation achieves an additional 10-fold concentration of vector with approx 75% recovery of infectious particles.

4. The S PCR assay presented here can detect 100 copies of target sequence in a background of 1 μg of genomic DNA by ethidium bromide stained gel and 1 copy of target sequence by Southern hybridization.

References


Methods for Efficient Retrovirus-Mediated Gene Transfer to Mouse Hematopoietic Stem Cells

John W. Belmont and Roland Jurecic

1. Introduction

A variety of genetic and acquired diseases could conceivably be treated by gene therapy targeted to hematopoietic stem cells (HSC). Inevitably, the effort to develop reliable methods of gene transfer into stem cells has raised many questions about their biology and role in the development and maintenance of hematopoiesis. As a result, we currently have a convergence of research goals in the areas of stem cell biology and gene therapy. Murine models for stem cell transduction have played a useful role in establishing two basic principles: retroviral vectors can transduce pluripotent self-renewing hematopoietic stem cells and retroviral vectors can express foreign gene products in the differentiated progeny of stem cells. Murine models also have allowed the identification of several key factors that allow efficient transduction of stem cells and each of these is dealt with here. However, methods for stem cell transduction that are effective with mouse cells have only been partially successful in dog, nonhuman primate, and human models. Whereas scale-up of stem cell transduction procedures for human applications will present unique technical problems, mouse models may yet provide further insight into the mechanisms of efficient stem cell gene transfer that can then be used to design enhanced and reproducible protocols.

1.1. Developmental Hierarchy of Murine Hematopoietic System

Hematopoiesis is an ongoing developmental process by which large numbers of mature blood cells with very specific functions and limited life-spans are continually replenished. Experiments using retroviral integration markers
have demonstrated that all blood cell types in mice originate from a common cell termed pluripotent hematopoietic stem cell (HSC) and provided some of the most rigorous evidence available for the existence of a transplantable stem cell (1-5). The HSC are defined functionally by their potential to regenerate and maintain all lineages upon transplantation into lethally irradiated or immunodeficient mice and are thus also called long-term repopulating (LTR) cells. Stem cells arise during embryonic development from the extraembryonic mesoderm as the yolk sac blood islands form. They then migrate to the fetal liver (where hematopoiesis takes place later during in utero development) and finally, take up residence in the bone marrow (BM), where they continue to survive in adulthood. The hematopoietic system is maintained by self-renewal and differentiation of HSC into uncommitted and committed progenitor cells, which in turn give rise to mature blood cells. Hematopoiesis is regulated by an intricate network of interactions between hematopoietic cells, cells of the hematopoietic microenvironment (stromal cells), and various growth factors. Proliferation, differentiation, and functional maturation of all hematopoietic cell types seem to be regulated by various hematopoietic growth factors (HGFs), which include interleukins (ILs), colony stimulating factors (CSFs), stem cell factors (SCF), leukemia inhibitory factors (LIF), erythropoietin (Epo), and other factors (interferons, transforming growth factors, tumor necrosis factors) (6-10). The establishment of efficient protocols for gene transfer into murine hematopoietic cells as well as the assessment of gene transfer efficiency have depended on various in vitro and in vivo assays for hematopoietic progenitors and LTR cells.

1.2. In Vitro Models for Murine Hematopoiesis

1.2.1. Colony Assays for Mouse Progenitors

In the mid 1960s, semisolid culture techniques that allow the clonal growth of murine BM cells with added growth factors were developed (11). These assays for colony forming cells (CFC) provided evidence for the presence of myeloid and lymphoid progenitor cells that are able to produce single and multilineage colonies of differentiated cells. Lineage-committed progenitors that can be demonstrated by CFC assays include progenitors for granulocytes (CFU-G), macrophages (CFU-M), erythrocytes (CFU-E), granulocytes and macrophages (CFU-GM), B-lymphocytes (CFU-B), and multilineage progenitors for granulocytes, erythrocytes, macrophages, and megakaryocytes (CFU-GEMM or CFU-Mix) (10,11). Further modifications of the basic CFC assay have been developed that allow assessment of more primitive or noncommitted progenitor cell populations. Blast cell colony assays (CFU-BI) detect colonies consisting of cells that are morphologically immature and, when
replated, give rise to secondary colonies of various lineages including more blast cell colonies (12). The high proliferative potential colony forming cell (HPP-CFC) assay detects very large colonies (>0.5-mm diameter) at 10–12 d of culture, formed by rare cells from normal or 5-fluorouracil-treated bone marrow. Recent studies indicate that HPP-CFC cells share many of the features of HSC (13). All these CFC assays have allowed the identification, purification, and cloning of numerous cytokine regulatory proteins, specific for growth of these colonies. They have also proven useful in analysis of gene transfer efficiency into murine committed and primitive progenitors. Individual colonies, derived from transduced BM cells, can be analyzed by PCR for proviral integration and for expression by other means to reflect retrovirus infection efficiency. For example, cells transduced with a neo-containing retrovirus can be selected in G418 for expression of the provirus. Other assays applicable to study of provirus expression in individual colonies include: analysis of isolated mRNA by reverse-transcription to cDNA and subsequent PCR (RT-PCR), microenzyme analysis, and immunocytochemistry on cytospin slides.

1.2.2. Long-Term Culture of Murine Bone Marrow

Long-term bone marrow cultures (LTBMC) have been particularly useful in the study of hematopoiesis, as the complex microenvironment of mammalian bone marrow precludes accurate and intensive analysis of this process in vivo. When cultured together, hemopoietic cells and marrow stromal cells can support hematopoiesis in vitro in the absence of added growth factors (14–19). Stromal cells sustain hematopoiesis by cell–cell interactions, cell–matrix interactions, and secreted growth factors (7–10). In LTBMC, cellular kinetics is in a dynamic equilibrium. For several months the loss of committed progenitors and mature cells through differentiation and cell death is balanced by constant differentiation of more primitive uncommitted progenitors. Thus, LTBMC share many characteristics with in vivo hematopoiesis and may be considered as an in vitro counterpart of the bone marrow. Simple manipulation of the culture conditions can be used to influence the type of hematopoiesis that is supported (e.g., myelopoiesis vs lymphopoiesis). The myeloid LTBMC selectively supports growth and differentiation of myeloid pluripotent and committed progenitors (CFU-S, CFU-GEMM, CFU-GM cells) as evidenced by their transplantation into irradiated animals and by CFC assays. Although myeloid cultures do not contain cells with lymphoid surface markers, they do contain lymphoid precursor cells, as evidenced by their ability to reconstitute B- and T-lymphocytes in irradiated animals or B-cell progenitors (CFU-B) in immunodeficient CBA/N and SCID mice (14–16). The lymphoid LTBMC selectively supports growth and differentiation of B-lineage cells from very early precursors to membrane IgM-bearing B-cells. All available evidence
indicates that lymphoid LTBMC are restricted to B-lymphoid differentiative pathway, since T-cells and cells of the myeloid lineage are undetectable as assessed by immunofluorescence and in vivo reconstitution experiments in immunodeficient or lethally irradiated mice. Furthermore, lymphopoiesis ceases and myelopoiesis does not establish upon transfer of established lymphoid cultures to myeloid culture conditions (17–19). In both LTBMC systems, the establishment and maintenance of active hematopoiesis depends on clonal proliferation and differentiation of primitive hematopoietic cells called long-term culture-initiating cells (LTC-IC) (20,21). The kinetics of lymphoid LTBMC establishment have indicated that cells initiating hematopoiesis in lymphoid cultures are primitive lymphoid precursors, which are closely related to pluripotent stem cells (22). Long-term bone marrow cultures have played an important role in the establishment of protocols for cell-free retroviral vector transduction of LTC-IC cells and HSC, which included autologous or heterologous stromal support.

1.3. In Vivo Models for Murine Hematopoiesis

Although in vitro systems have proven useful in the analysis of committed and primitive progenitors, it has been difficult to correlate the growth of cells in culture with the maintenance and differentiation of the most primitive progenitors and stem cells. Analysis of these cells has depended on BM transplantation into lethally irradiated recipients. Three in vivo assays have been established and widely applied in the study of primitive progenitors and HSC (6). The first assay detects the myeloid-restricted precursors, termed colony forming unit-spleen (CFU-S) cells, that home to the spleen of recipient animals after injection (23). The quantitative nature of this assay is based on a direct linear relationship between splenic nodules or colonies present 8–12 d after transplantation and the number of cells injected and the clonal origin of colonies. The second assay, termed marrow repopulation assay (MRA), detects the population of pre-CFU-S cells or marrow repopulating CFU-S d 12 cells (MRA-CFU-S_{12}) (24). These cells are present in the marrow 12–14 d after initial transplant and are defined by their ability to generate CFU-S_{12} in a secondary recipient. The MRA assay provides semiquantitative results that can be used to compare different populations for hematopoietic repopulating ability. These two assays have found broad application in retroviral vector-mediated gene transfer studies. They have been used extensively to identify conditions that enhance transduction of primitive hematopoietic progenitors by causing their proliferation or expanding their pool (25,26).

The hematopoietic stem cell activity, defined by long-term repopulation of all hematopoietic lineages in irradiated animals, has been analyzed with long-term reconstitution (LTR) and competitive repopulation assays (CRA) (27,28).
In these assays, the test population is transplanted into lethally irradiated animals without (LTR assay) or with (CRA assay) an excess of syngeneic BM cells whose LTR capacity has been markedly reduced by two previous cycles of serial transplantation. The latter compromised population contains a normal frequency of mature clonogenic progenitors; they provide short-term support for recovering transplant recipients, but, in the later stage of engraftment, exert very little competitive pressure on test population. Genetically marked cells (isolated from transgenic mice) or congenic marker systems (Ly-5) are used to detect progeny of test population. Three to six months after transplant, peripheral blood of reconstituted animals is periodically analyzed for lineage-specific markers and donor vs host cells. As a final test for stem cell activity, bone marrow of reconstituted primary recipients can be transplanted into secondary recipients and ability of test population to generate all hematopoietic lineages in secondary hosts is analyzed. However, since the above assays are not quantitative, it is unclear whether reconstitution originates from individual pluripotent cells or various primitive progenitors (6). In comparison, stem cell marking by retroviral vector insertion offers the unique advantage of allowing determination of clonal relatedness among groups of repopulating cells. To quantitate LTR cells in BM populations enriched for HSC, limited numbers of genetically marked cells (1–20 cells) are coinjected with syngeneic BM cells into lethally irradiated animals and level of repopulation by marked cells is analyzed as described (6).

To study reconstitution in murine transplantation models, various properties unique to the repopulating cells have been exploited. In addition to using a Y-chromosome marker to study repopulation in sex-mismatched transplants, various polymorphic loci on congenic backgrounds (hemoglobin, glucose-phosphate-isomerase, Thy-1, and Ly-5 antigens) have proven useful for analyzing the differentiation of repopulating cells into mature hematopoietic lineages. Along with CFU-S and LTR assays, these markers have been particularly informative in the development of stem cell enrichment protocols and functional analysis of enriched populations.

1.4. Enrichment of Murine Hematopoietic Stem Cells

Enrichment of murine HSC has depended on development of fluorescence-activated cell sorter (FACS) separation techniques, based on cell surface antigen expression, lectin binding, and uptake of fluorescent vital dyes. A rare population of mouse BM cells (0.05%) was isolated by FACS, which expresses low levels of Thy-1 antigen (Thy-1\(^{lo}\)), high levels of Sca-1 antigen (stem cell antigen-1, a member of the Ly-6A/E antigen family) and is lineage-negative (Lin\(^{-}\)), i.e., it does not express markers characteristic of B-cells, T-cells, granulocytes, and myelomonocytic cells. Small numbers of Thy-1\(^{lo}\)Lin\(^{-}\)Sca-1\(^{+}\) and
Lin−Sca-1+ cells can generate long-term repopulation of both lymphoid and myeloid lineages, protect mice from lethal irradiation, and further repopulate secondary recipients (29–33).

Further fractionation of Thy-1loLin−Sca-1+ and Lin−Sca-1+ cells on the basis of c-kit proto-oncogene (receptor for stem cell factor—SCF) expression has shown that long-term repopulating ability is essentially restricted to cells expressing the Sca-1 antigen and c-kit, but has also underlined functional heterogeneity of both enriched populations (34). Studies of long-term repopulation by Thy-1loLin−Sca-1+ cells have confirmed that this population contains in vitro colony-forming cells (CFC), d-12 CFU-S, long-term repopulating (LTR) cells for both the myeloid and lymphoid lineages and cells capable of long-term repopulation of only the lymphoid lineage (33). Cell cycle analysis of Thy-1loLin−Sca-1+ cells has shown that a significant proportion (18–22%) of these cells are actively proliferating during steady-state hematopoiesis and that this subpopulation exhibits reduced stem cell activity (35). Recent purification and functional analysis of Lin−Sca-1+ cells that express carbohydrate binding sites for the lectin wheat-germ agglutinin (WGA) have demonstrated that this population (Lin−Sca-1+WGA+ cells), although highly enriched for LTR and CFU-S cells, is still functionally heterogeneous (36,37). In spite of substantial progress in purification of cell populations highly enriched for stem cell activity, novel markers are needed to more precisely define the phenotype of LTR cells and separate them from the rest of the primitive hematopoietic cells. However, purification of rare BM populations has enabled retroviral transduction and assessment of gene transfer efficiency into highly enriched primitive hematopoietic progenitors and stem cells.

1.5. Ex Vivo Maintenance of Murine Primitive Hematopoietic Cells

The CFC assays and procedures for enrichment of progenitor and stem cells have enabled the study of growth factor requirements of these rare cells that might lead to culture conditions for their propagation without differentiation—a key goal in gene therapy research. The analysis of in vitro response of phenotypically and functionally defined murine progenitor and stem cell populations to various growth factors has shown that Thy-1loLin−Sca-1+ and Lin−Sca-1+ cells are generally not responsive to any single factor tested but require a combination of factors (IL-1, IL-3, IL-6, G-CSF, M-CSF, GM-CSF, SCF) for maximal colony formation in CFC assays (32,34,38–40). These studies have revealed a synergistic pattern of action of multiple growth factors and suggested that primitive murine hematopoietic cells need multiple GF signaling for entering the cell cycle and initiation of differentiation. Furthermore, culture of Thy-1loLin−Sca-1+ and Thy-1loLin−Sca-1− cells at limiting dilution...
in Dexter's myeloid LTC has demonstrated that only this rare population contains cells with LTC-IC activity (41).

Further efforts, directed toward determining the combination of factors that support survival and cycling of primitive progenitors and stem cells without differentiation have demonstrated that SCF in synergy with IL-3 and IL-6 induces expansion of CFC and CFU-S cells in vitro and in vivo (42–44). Recently it was shown that SCF, IL-6, and Epo induce amplification of Sca-1+Lin-WGA+ cells in serum-free cultures with maintenance of long-term reconstituting cells (37). Furthermore, the analysis of mice deficient for LIF has generated evidence that LIF is required for the survival of HSC, but not their terminal differentiation (45).

1.6. Efficient Gene Transfer to Mouse Hematopoietic Stem Cells

The low frequency of HSC in the bone marrow (one in \(10^4\)–\(10^5\) nucleated cells) presents a difficult problem for gene transfer, making efficient transduction of such a rare subpopulation dependent on near quantitative gene transfer. The first demonstration that hematopoietic progenitors could be transduced with a retroviral vector was provided by Joyner et al. (46). In those experiments, a vector carrying the bacterial Tn5, neomycin phosphotransferase (neo) gene was shown to confer G418 resistance to murine hematopoietic colonies. Williams et al. (47) and Dick et al. (48) extended these studies by demonstrating that very primitive precursors could also be transduced by similar vectors. After some early problems with the design of vectors that were permissive for expression of foreign genes, numerous publications have demonstrated conclusively that retroviral vectors can express genes at physiologically relevant levels in the differentiated progeny of stem cells (49–51).

Gene transfer to murine hematopoietic cells has been achieved using retroviral vectors containing a variety of genes, including the bacterial neomycin-resistance gene (neo'), human dihydrofolate reductase (DHFR), and multidrug resistance-1 (MDR1) genes, human β-globin, human adenosine deaminase (ADA), CD18, and glucocerebrosidase (GC) (49–51). Several groups have demonstrated retrovirus-mediated gene transfer of human ADA into mouse HSC in the absence of replication-competent retrovirus. Stable and potentially therapeutic levels of in vivo expression of the human ADA were achieved in all hematopoietic tissues of recipient mice (52,53). Although some early studies suggested that expression in stem cells was limited by regulation of the retroviral long terminal repeat promoters, multiple examples have subsequently shown that such simple vectors do express introduced genes. These studies have demonstrated that the single most important factor limiting long-term expression is the efficiency with which stem cells are transduced.
A number of variables appear to be important for efficient transduction of murine primitive progenitors and stem cells, such as: use of very high titer viruses (>\(10^6\) infectious particles/mL), 5-fluorouracil pretreatment of the donor animals prior to BM harvest, gradient separation of BM cells for enrichment of stem cells, coculture of the target BM cells with the retrovirus-producing cells or viral supernatant infection of BM cells on stromal cell layers, and inclusion of hematopoietic growth factors (recombinant IL-1, IL-3, IL-6, SCF, LIF) in the culture media before and/or during transduction (54–61). The presence of growth factors, which increases survival and number of cycling progenitors and stem cells, appears to be critical for efficient transduction and expression of the transgene in the progeny of transduced progenitors and stem cells. However, the optimal set of growth factors that would induce cycling of primitive hematopoietic cells without their differentiation in vitro has not been determined yet. Recent reports on efficient transduction of highly enriched murine long-term repopulating cells and spleen-colony forming cells (CFU-S) were the first to demonstrate the feasibility of introducing heterologous genes into purified HSC, an approach that could represent a major advance in the field of gene therapy (62,63).

2. Materials

1. Methylcellulose mix (final concentration): MC stock (0.8–0.9%)—see Section 3.1.2 procedure below for method of preparation, fetal calf or calf serum (30%), deionized bovine serum albumin (1%), 2-mercaptoethanol, tissue culture grade (10^{-4}M), L-glutamine (0.29 mg/mL) (see Note 1).

2. Colony stimulating factors (CSF). 3 U/mL Epo with 100 U/mL rIL-3 or 10% (v/v) WEHI-3B cell-conditioned medium (CM)

3. Myeloid long-term culture media: Alpha medium supplemented with 400mg/L L-glutamine, 40 mg/L i-mositol, 10 mg/L folic acid, 12.5% horse serum*, 12.5% FCS* (each serum should be used without heat inactivation), \(10^{-6}M\) 2-mercaptoethanol, \(10^{-6}M\) hydrocortisone sodium succinate in alpha medium, added immediately prior to use. Do not use after 7 d (see Notes 2 and 3).

4. Bone marrow/retrovirus transduction culture medium (BMM): IMDM (base media), 10% BCS, 10 mg/mL BSA, 0.0017% \(\alpha\)-thioglycerol (Sigma, St. Louis, MO; tissue culture grade), 4 \(\mu\)g/mL polybrene, 100 U/mL penicillin, 100 \(\mu\)g/mL streptomycin (Gibco-BRL, Gaithersburg, MD), 10 U/mL recombinant murine LIF (commercial source).

5. Percoll discontinuous density gradient: Prepare Percoll solutions with densities of 1.10, 1.09, 1.07, and 1.06 g/cm^3 according to the protocol recommended by the manufacturer (Pharmacia, Uppsala, Sweden). Adjust the pH of solutions to pH 7.0 and osmolarity to 300 ± 5 mOsm/kg. Keep all solutions sterile and at 4°C.

*Standardize by pretesting selected lots for the ability to sustain clonogenic output from a single inoculum of normal marrow for 8–12 wk in culture as an appropriate endpoint.
6. Monoclonal antibodies (MAb) specific for murine T-lymphocytes (CD4, clone GK1.5; CD8, clone 53.6.7.), B-lymphocytes (B220, clone RA3-6B2), macrophages (Mac-1, clone MI/70), granulocytes (Gr-1, clone RB6-8C5), Thy-1.1 (clone 19XE5), and Thy-1.2 antigen (clone 53.2.1) are widely available from commercial sources. Monoclonal anti-LydA.2 (Sea-1, clone E13-161-7) could now be obtained from commercial sources or by inquiries to Dr. I. L Weissman (Stanford University, Palo Alto, CA).

7. Equipment:
   b. Small animal irradiator: Many investigators prefer the Gammacell 40 137Cs source because of dose rate and field homogeneity (Nordion, Ontario, Canada).

8. Mice can be purchased SPF from reliable vendors. Recent screening information should be enclosed with each shipment or be available on request. Mice can be maintained SPF in a monitored-barrier facility or in a conventional facility with adequate precautions. Mice to be rendered immunodeficient by lethal total body irradiation (TBI) should be housed in autoclaved microisolator cages with autoclaved bedding, food, and water. Hepa-filtered ventilated cage racks and special changing hoods provide a necessary, additional level of safety in a conventional facility with endemic mouse viruses. The colony should be monitored quarterly for mouse viral pathogens (mouse level II complete antibody profile, Microbiological Associates, Rockville, MD), bacterial pathogens, and endo- and ectoparasites.

3. Methods

3.1. Committed Progenitor Colony Assays

3.1.1. Bone Marrow Cell Preparation

1. Sacrifice mice by cervical dislocation and secure to dissection board.
2. Flood the hind limbs with 70% ethanol and reflect the skin.
3. Reflect muscles at their attachments; cut away as much as possible with scissors.
4. Separate femur and tibia from the body at the hip joint and at the base of the feet.
5. Remove muscles with paper tissue, then place in a dish with IMDM and antibiotics on ice.
6. Once all bones have been collected, transfer to fresh IMDM in the culture hood.
7. Remove bone epiphyses with scissors and using a syringe (25–27-gage needle) filled with IMDM plus 10% fetal or bovine calf serum; expel the marrow.
8. Prepare single-cell suspension by gentle repeated pipeting. Perform viable cell count by Trypan blue exclusion. Wash 2X in IMDM at 1000g for 10 min. From one mouse, 4–5 x 10^7 bone marrow cells can be obtained.

3.1.2. Preparation of 2% Stock Methylcellulose

1. Prepare 2X IMDM by dissolving a 1-L packet plus 3.25 g NaHCO₃ in a total volume of 500 mL with water. Sterilize by filtration through a 0.22-μm filter.
2. Add 20 g methylcellulose (Methocel MC 4000 mPa.s, Fluka, Ronkonkoma, NY) to 500 mL boiling ddH₂O in weighed, sterile 2-L Erlenmeyer flask. Reheat just to the boiling point and remove immediately from heat.

3. Cool to 40–50°C under running water and add the 500 mL of double-strength IMDM. Hold overnight at 4°C with continuous stirring. Check weight and adjust to 1000 g with sterile water if necessary.

4. Dispense in 40-mL aliquots in 50-mL conical tubes. These can be stored at −20°C indefinitely. Avoid frost free freezer. Thaw at room temperature.

3.1.3. Culture Preparation

1. Dilute cells in IMDM plus CSF mixture to 5 × 10⁴/1-mL culture.
2. Prepare cultures in triplicate. Mix cells, supplements, and methylcellulose in a 3-mL syringe fitted with an 18-gage needle. Avoid bubbles.
3. Dispense 1 mL in the middle of a 35-mm culture dish, rock gently, or rotate to spread.
4. Place two dishes of cells plus one dish of sterile water in 10-cm Petri or tissue culture dish. Transfer to incubator, fully humidified, 5% CO₂, 37°C.
5. Examine at 7, 14, and 21 d with an inverted microscope.

3.1.4. Colony Identification

Colonies are defined as >50 cells, although their size varies widely (an average-sized colony has 200–500 cells). Size is dependent on the proliferative status of the marrow and the combination of growth factors added. With practice, individual colony types can usually be determined by microscopic inspection. Confirmation can be accomplished by individual colony picking and microscopic inspection of cytospin slide preparations stained with Wright-Giemsa stain. The following is a list of common colony types and suggested stains for positive identification.

1. BFU-E: Erythroid burst-forming unit, erythropoietic progenitors more primitive than CFU-E, which generates colonies containing multiple erythroblast clusters in culture (mature BFU-E: 3–8 clusters, primitive BFU-E: >8). Positive staining with benzidine.
2. CFU-G: Granulocyte, multishaped nuclei representing the varying stages of differentiation from myelocyte (ring-shaped nuclei in mice) to the characteristic polymorph. Positive myeloperoxidase staining.
3. CFU-M: Macrophages, round eccentric nucleus, often with cytoplasmic evidence of phagocytosis. Positive with nonspecific esterase staining.
4. CFU-GM: Contains both granulocytes and macrophages.
5. CFU-Mix: More primitive precursor that gives rise to three or more of the following cell types: granulocytes, erythroid cells, macrophages, mast cells, eosinophils, and megakaryocytes. Mast cells contain metachromatic granules that stain positive with Astra Blue, whereas eosinophils stain bright green with Luxol Fast Blue.
3.1.5. Gene Transfer Analysis for Murine CFC Cells

1. Select G418 or hygromycin B-resistant colonies if the vector encodes neo or hygromycin phosphotransferase. Without selection, pick well-isolated individual colonies, transfer into sterile Eppendorf tubes, wash with PBS (2X).
2. Resuspend in 10 μL of sterile ddH₂O, boil for 2 min, and briefly centrifuge. Use the supernatant as a source of DNA for PCR analysis.

3.2. Myeloid Long-Term Culture of Mouse Bone Marrow

3.2.1. Establishment of Dexter’s Myeloid LTBMC

1. Seed 2–3 × 10⁷ BM cells in 10 mL of myeloid-LTC media in T-25 flask (Costar, Cambridge, MA; 0.2-μm vented filter flask).
2. Maintain at 37°C, 5% CO₂ in air for 3 d, then transfer to 33°C, 5% CO₂ in air.
3. After 7 d and then at weekly intervals, remove half of the media and nonadherent cells and replenish the cultures with fresh media to 10 mL. Confluent hematopoietically active stromal layers should be established after 2–3 wk.
4. Cultures should maintain active hematopoiesis for at least 2–3 mo. Hematopoietic activity can be monitored by output cell number and CFC assays.

3.2.2. Preparation of Stromal Feeder Layers for Culture of Enriched or Transduced BM Cells

1. Establish LTBMC in T-25 flasks or for limiting-dilution analysis in 96-well plates as described in Section 3.2.1.
2. After the adherent monolayer has become established, irradiate cultures with a total dose of 20 Gy in order to remove all endogenous hematopoietic activity. Maintain in LTC medium with weekly half-media change.
3. Seed sorted cells enriched for HSC activity at several graded concentrations and maintain the culture as described. For whole BM infected with recombinant retrovirus, seed 3 × 10⁶ cells/flask and maintain as described in Section 3.2.1.

3.3. In Vivo Assays for Progenitor and Stem Cells

3.3.1. Primary CFU-S, d 12 (CFU-S-12)

1. Irradiate recipient animals with total dose of 9.0–12 Gy, dependent on the strain of mice used. Lower dose rates (0.3–1.0 Gy/min) are preferred. Nontransplanted radiation control mice should live for 14–21 d if housing and radiation standards are adequate. The mice should be transplanted within 6–12 h of lethal TBI. Transplant lethally irradiated mice with 10⁵ whole marrow cells or 10⁵–500 sorted BM cells enriched for stem cell activity.
2. Inject cells (resuspended in phosphate-buffered saline, PBS) iv into the retro-orbital venous plexus with a blunt needle (in 50–100 μL vol) or into the lateral tail vein using a 25-gage needle (in a volume of up to 0.5 mL). Anesthesia is required for retro-orbital injection. Use a 50-mL conical tube containing a gauze dampened with methoxyflurane (Metofane) for the anesthetic chamber.
a. Mice anesthetize and recover quickly (<1–2 min)

b. Monitor anesthesia by toe pinch (withdrawal of limb) and respiration (rate and depth)

3. After 12 d, dissect the spleens
   a. Fix in Bourn’s solution, consisting of 75 mL saturated picric acid, 25 mL 37% formalin, and 5 mL concentrated acetic acid
   b. Enumerate colonies with the aid of a dissecting scope
   c. If interested in assessing gene transfer efficiency; carefully dissect large, discrete colonies (approx 10^6 cells) without fixation. Protein extracts for expression studies of the transferred gene and DNA for provirus analysis can be prepared. Whole marrow recovered after retroviral transduction will yield approx 10–20 CFU-S-12/10^5 cells transplanted.
   d. Spleens can also be fixed in neutral-buffered formalin, followed by sectioning and H&E staining to identify microscopic colonies and determine the cellular composition of all colonies

3.3.2. Marrow Repopulating Ability-CFU-S, d 12 (MRA-CFU-S-12)

1. Transplant lethally irradiated mice with 10^6 whole marrow cells. If using enriched cell populations, cell dose is dependent on the enrichment achieved. Quantitation of MRA can be approached by limiting dilution of the marrow inoculum in the primary recipients.

2. After 13 d, isolate the marrow from one femur.

3. Perform viable cell count and inject secondary lethally irradiated recipients with 10^5 cells

4. Maintain 12 d, then harvest spleens and count colonies or dissect as in Section 3.3.1.

3.3.3. Long-Term Reconstitution and Competitive Repopulation Assay

1. Transplant lethally irradiated mice with 10^5–10^6 whole marrow cells or 200–500 sorted cells of unknown stem cell content (test population). For competitive repopulation assay, transplant the equal number of “competitor” fresh bone marrow cells with known repopulating ability or compromised bone marrow cells. Quantitation of repopulating cells in “test” population can be further enhanced by transplanting graded numbers of cells while using the competitor population at a constant cell dose.

2. Allow mice to reconstitute for at least 6 mo.

3. Engraftment can be studied by measuring the percentage of mature cells in various lineages bearing the genetic marker of “test” population by flow cytometry. Alternatively, colony assays and/or CFU-S assays can be used, starting with marrow cells of the primary recipients. This is particularly helpful since it allows precise quantitation within the precursor pools.
3.4. Retroviral Vector Transduction of Murine HSC

1. Establish monolayer of irradiated virus-producing cells.
   a. Trypsinize from logarithmic growth phase (~70% confluent flasks). Wash cells and resuspend in growth media.
   b. Irradiate (20 Gy) in suspension (1–5 × 10^6/mL).
   c. Plate irradiated cells at 3 × 10^6 cells in 100-mm tissue culture dishes. Allow cells to adhere at least 4 h prior to addition of BM cells.

2. Remove media and add 3 × 10^6 BMC in 10 mL BMM to established virus-producing monolayers. Cocultivate for 72 h at 37°C, 5% CO₂.

3. Harvest marrow cells from dishes.
   a. Remove nonadherent cells and save.
   b. With fresh media, vigorously wash the fibroblast layers to dislodge adherent marrow cells.
   c. Allow fibroblast clumps to settle by gravity for 10 min.
   d. Repeat gravity sedimentation if necessary to remove clumps.
   e. Combine fractions, wash 2X in IMDM, prepare single cell suspension, and perform viable cell count.

4. Inject marrow cells into lethally irradiated syngeneic recipients.
   a. Cell number injected is dependent on assay conducted.
   b. For long-term transplant controls include: mice injected with the mock-infected marrow (cells incubated over packaging cells without vector) and radiation control mice.

5. Although transduction is vector-dependent, you may expect transduction efficiencies of 50–100% under these conditions, using a vector with a titer of ≥10^6 U/mL.

3.5. Enrichment of Murine Hematopoietic Stem Cells

The protocol described herein is a modification of the method for enrichment of mouse Lin⁻Sca-1⁺ cells (36). It includes two steps. The first is a selection of low density marrow cells using a discontinuous Percoll gradient. This step removes the majority of granulocytic cells and thus reduces the sorting time. The second step is sorting of cells on the basis of size, cellular heterogeneity, and fluorescence intensity.

1. Prepare bone marrow cells from 5–10 mice (8–12 wk old) as described. Form the Percoll gradient by layering sequentially 2 mL of 1.10, 1.09, and 1.07 g/cm³ solution.

2. Resuspend up to 5 × 10^7 cells in 2 mL of low density (1.06 g/cm³) Percoll solution and load on top of a three-layer discontinuous Percoll gradient in round-bottomed polystyrene tubes. Centrifuge at 1000g, 4°C for 25 min.

3. Harvest the low density cells at the 1.06/1.07 g/cm³ interface and wash twice in cold 0.15M NaCl. Count the viable cells (yield should be ~13% of the starting number of cells) and resuspend in PBS with 5% FCS and antibiotics (10^7 cells/mL).

4. Incubate the cells with a cocktail of lineage-specific rat MAb, directly conjugated with FITC. After 35-min incubation on ice, wash the cells twice (800g for 10 min) and resuspend at the same density.
5. Incubate the cells with rat anti-Ly6.2A (Sca-1) for 35 min on ice. Wash the cells twice with PBS and incubate for another 30 min with phycoerythrin (PE)-conjugated F(ab')2 goat antirat IgG and repeat the wash. Count the viable cells and resuspend at $3 \times 10^6$/mL in PBS with 5% FCS and antibiotics.

6. Analyze the cells on a presterilized dual laser FACStarPlus or FACS-Vantage instrument (Becton Dickinson Immunocytometry Systems, Rutherford, NJ) and set the gates for four-parameter sorting. Select the cells for sorting on the basis of intermediate forward scatter (to exclude small lymphocytes and debris), low side scatter (to exclude highly granular cells), low levels of FITC fluorescence (lineage negative cells, Lin-), and high levels of PE fluorescence (Sca-1+ cells). Keep the cells at 4°C.

7. Sort the cells at a rate of 1000–2000 cells/s and collect them in cold PBS containing 10% FCS and antibiotics. After completion of the sort (yield is approx 0.05–0.1% of starting cell number; the purity and viability of sorted cells should be about 90% or more), wash the cells twice (800g for 10 min), count, and resuspend as required for assays (see Note 4).

4. Notes

1. Methylcellulose (MC) as the semisolid support medium for colony assays has several advantages. Foremost is the relative ease of picking colonies for replating, DNA prep, or microscopic examination. Methylcellulose is less viscous at room temperature than at 37°C but requires volume displacement pipeting for accuracy. Methylcellulose preparations for mouse progenitor assays are available from StemCell Technologies, Vancouver, BC.

2. Different lots of serum and BSA should be pretested and selected for the best growth of colonies in MC and establishment and maintenance of LTBMC.

3. Only deionized-distilled water (ddH2O) should be used to prepare cell culture media and other solutions. Plasticware is preferred for pipets and other supplies. All glassware should be thoroughly rinsed with ddH2O prior to sterilization. Low level contamination with organic solvents or soaps will cause poor cell growth.

4. Several parameters that affect sorting should be mentioned. The purity, yield, and viability of sorted cells are the most important parameters to control. The purity of the sort is usually assessed by reanalyzing the sample of sorted cells and determining the percentages of all events that pass through the sorting windows. The yield of cells is determined practically, as losses often occur during sorting, thus making the number of sorted cells reported by instrument inaccurate. By knowing the starting concentration of cells and the percentage of cells gated, the theoretical yield can be calculated. The number of sorted cells can then be measured and the actual yield calculated. The ultimate goal of sorting is to obtain a highly purified and viable (~95%) cell population. Among the many factors that will affect viability of sorted cells, the most important are the temperature and the collecting medium. During the entire procedure, cells should be kept at 4°C in IMDM or PBS supplemented with 5–10% FCS and antibiotics.
Retrovirus Transfer to Stem Cells

References


Methods for Retrovirus-Mediated Gene Transfer into Primary T-Lymphocytes

Michel Sadelain

1. Introduction

Recombinant retroviruses are efficient vectors for introducing genes into many mammalian cell types. They are useful in the context of clinical as well as experimental applications, owing to their ability to generate high-titer and helper-free viral stocks. Retroviral vectors are especially appropriate for the transduction of primary lymphocytes, because gene transfer is mediated by nonimmunogenic vectors and stable vector integration in the target genome is achieved. Stable integration in cells undergoing clonal expansion ensures that the foreign genetic material will be faithfully transmitted to the cells’ progeny.

1.1. Factors Controlling Retrovirus-Mediated Gene Transfer

Gene transfer mediated by viral vectors follows the molecular pathway of the natural infectious process. Therefore, the factors enhancing or restricting natural infection will influence retrovirus-mediated gene transfer efficiency. The basis for the design of retroviral vectors and packaging cells are briefly reviewed in Chapters 3 and 4. The critical factors relevant to retroviral infection include:

1. Cell division: Cell division is required for efficient retrovirus-mediated gene transfer (1,2). Therefore, postthymic T-lymphocytes must be activated and cycling in order to be transduced using current Moloney-murine leukemia virus (MLV)-based vectors.

2. Viral receptor expression: The receptors for MLV particles (3–6) are expressed by T-lymphocytes. However, the receptor distribution is poorly characterized with respect to T-cell subset, cell cycle, and activation status.
3. External factors: Several factors outside the target cell are able to interfere with retroviral infection. The presence in the extracellular compartment of nonspecific mediators, such as complement (7) and interferons (8), or specific factors, such as neutralizing antibodies (9) and soluble receptor-like moieties (10), will adversely affect the infectious process. These factors can act on a number of the steps necessary for the efficient generation of a provirus, from the binding of the viral particle to the target cell receptor to the chromosomal integration of the reverse-transcribed viral genome. Virally induced resistance to infection, or viral interference, can operate on the receptor itself, the cytoplasmic membrane, or inside the cell. All of these mechanisms can contribute to reducing the efficiency of retrovirus-mediated gene transfer.

Thus, ex vivo retrovirus-mediated gene transfer is only possible in cycling T-lymphocytes expressing the viral receptor and under conditions that elude extracellular and intracellular mechanisms of resistance to infection.

1.2. Considerations for the Culture and Retroviral Infection of Primary T-Cells

Lymphocyte division can be elicited by numerous stimuli, either by engaging the T-cell receptor with superantigen or processed antigen and MHC, in conjunction with adequate costimulatory signals, or by various cross-linking agents, which include lectins and antibodies. However, it is difficult to greatly expand primary lymphocytes and maintain them in culture for sustained periods of time, whatever the initial mitogenic signal(s) (11). Optimal conditions for the expansion of antigen-reactive clones require periodic antigen pulsing and the addition of lymphokine and adequate antigen-presenting cells. Otherwise, prolonged culture of polyclonal lymphocytes will typically lead to T-cell subset imbalance, uncontrolled repertoire bias, phenotypic alterations, and postactivation apoptotic cell death.

The addition of high-titer retroviral stocks to freshly harvested lymphocytes results in very low infection efficiency. Mitogenic stimulation of the lymphocytes before exposure to the viral particles, as well as the addition of a polycation such as polybrene or protamine sulfate, is an absolute prerequisite for achieving significant gene transfer (12–17). Whereas retroviral supernatants are sufficient for achieving significant gene transfer in human T-lymphocytes (>10% gene transfer efficiency), supernatants only yield marginal gene transfer (<1%) in primary murine T-lymphocytes. Several factors affect the infection efficiency:

1. T-cell activation: Numerous stimuli can trigger mitosis in lymphocytes. The timing of the exposure to the virus relative to the initiation of T-cell activation is a critical variable that varies with the activation conditions. In the procedure described below, a 48-h interval between the two events is optimal for human lymphocytes.
2. Culture conditions. Cell densities must be optimized for lymphocyte survival in vitro. Cocultivation of preactivated lymphocytes with high-titer producers under well-defined conditions is more efficient than supernatant-mediated infection (see Note 1). Cocultivation is essential to achieve substantial gene transfer in murine primary lymphocytes (>10% gene transfer efficiency; see Note 2).

3. Medium composition: Certain elements must be present throughout the infection procedure: a suitable, prescreened serum, interleukin-2, and an enhancer of infection, such as polybrene, protamine sulfate, or polycationic liposomes. Polybrene increases infection efficiency but its toxicity reduces lymphocyte recovery. Thus, the lymphocytes should be grown in their preferred medium before and after exposure in the infection step. The level of receptor expression can be affected by the medium composition, at least in some circumstances (18).

4. Retroviral particles: Amphotropic particles may be used for both murine and human target cells. Ecotropic particles can only be used to infect the former. Some recent data suggest that particles coated with the GaLV retroviral envelope might be more effective than particles bearing the amphotropic envelope for the infection of human T-lymphocytes (M. Sadelain, unpublished observations, and R. A. Morgan, Bethesda, MD, personal communication).

1.3. Gene Transfer, Gene Expression, and Vector Design

Gene transfer can be quantified directly by analyzing the target-cell genomic DNA or indirectly by assaying for gene expression. Genomic DNA analysis based on a careful vector copy number determination is an accurate way to quantify gene transfer (see Chapter 4). Gene transfer efficiency can also be estimated using gene expression assays, provided that gene expression can be readily determined on a per cell basis (see Note 3). This quantification may be achieved by either subcloning the infected population by limiting dilution and scoring the fraction of positive wells (by DNA, RNA, or protein analysis), or more directly by immunofluorescence when a marker is amenable to fluorescent detection, e.g., a cell-surface marker or Escherichia coli β-galactosidase. An ideal marker is biologically inactive or neutral in lymphocytes, nonimmunogenic, and easily detectable by fluorescence-activated cell-sorting (FACS). An example of gene transfer analysis by cell-surface phenotyping is shown in Fig. 1.

When the gene of interest must be expressed in every lymphocyte with a high degree of certainty, it is necessary to introduce a selectable marker or a screening marker into the vectors. Selectable markers, such as neomycin phosphotransferase, are effective but lead to all the caveats mentioned above. As we and others reported earlier (19,20), the use of cell-surface markers as screening markers is superior for purifying lymphocytes and hematopoietic cells.
Fig. 1. Retrovirus-mediated gene transfer in human primary T-lymphocytes: analysis of transgene expression by cell-surface phenotyping. On the Y axis, CD3 signal; on the X axis, cell-surface marker signal. (A) T-cells infected with a control vector; (B) T-cells infected with a vector encoding a modified low-affinity human nerve growth factor receptor (LNGFR*) as the reporter molecule. LNGFR is mutated in the extracellular domain to complement a large cytoplasmic domain deletion that alone fails to abrogate receptor function (25, Grallardo, M., et al., manuscript in preparation). The percentage indicated in the upper-right quadrant is the percent of LNGFR*-positive cells among all cultured T-lymphocytes. The infection procedure is described in the text. The viral supernatant is harvested from a PG13 (24) producer cell line that was generated as described in Chapter 4.

because the transduced cells can be sorted by panning, column or magnetic separation, or FACS. Thus, dual-expression vectors coexpressing the gene of interest and a cell-surface marker are necessary. Dual promoter vectors are often used, but they are prone to transcriptional interference (21,22). In the event of such interference, the assumption that the gene of interest is expressed in a cell identified as expressing the marker gene is tenuous. The average frequency of uncoupled expression must be experimentally determined for any vector. The internal ribosomal entry site (IRES) from Picornaviridae, which allows for the generation of bicistronic mRNA, can be used to resolve this problem (see Chapter 4), if it is established that the IRES element is functional in T-lymphocytes.

Ideally, the retroviral vector should provide for a predictable and controlled level of transgene expression, one that is independent of the site of integration, constant over time, and eventually inducible or regulated. A discussion of these issues, however, is beyond the scope of this chapter. The Moloney MLV transcriptional control elements (12–14), which control gene expression in many retroviral vectors (see Chapter 4), as well as other constitutive promoters (15–17), have been shown to function in T-lymphocytes.
2. Materials

2.1. Reagents

1. RPMI-1640 (Gibco-BRL, Grand Island, NY).
2. Phosphate-buffered solution, PBS (Gibco-BRL).
3. Lymphoprep, Nycomed Pharma (Gibco-BRL)
4. Fetal bovine serum (FBS).
5. Penicillin, streptomycin (Gibco-BRL).
6. Phytohaemagglutinin (Murex Diagnostics, Norcross, GA). Stock in culture medium is stored at -20°C.
7. Polybrene (Sigma, St Louis, MO). Stock in phosphate-buffered saline is stored at 4°C.
8. Interleukin-2, human, step 1–purified, or recombinant.
9. hT medium: 10% (v/v) FBS, penicillin 100 U/mL, streptomycin 100 μg/mL in RPMI-1640.

3. Methods

The methods described here are established for freshly harvested human peripheral blood lymphocytes (see Note 4). High-titer (>10^6 infectious particles/mL) producer cell lines are generated from the packaging cells ψ-CRIP and PG13 (23, 24) as described in Chapter 4. All cell culture is carried out in humidified incubators at 37°C in a 5% CO₂ atmosphere.

The procedure described here is used in an experimental setting. It provides a conceptual framework and practical reference for methods specifically designed for gene transfer in antigen-specific T-cell clones or T-cell lines used in a clinical setting.

3.1. Infection of Primary Human T-Lymphocytes

1. Day 1: Freshly harvested peripheral blood cells are washed once in PBS, separated over a monolayer of Ficoll (2500 rpm for 20 min at 25°C), and washed twice. The cells are cultured at a density of 1–2 ¥ 10^6 cells/mL in hT medium containing PHA at 0.5 μg/mL.
2. Day 3: The nonadherent cells are harvested, washed once, and resuspended at a density of 10^6 cells/mL in hT medium containing human IL-2 at 10 U/mL. An equal volume of high-titer viral supernatant is admixed along with polybrene (4 μg/mL final concentration). The lymphocytes are cultured for 24 h.
3. Day 4: The cell cultures are harvested, washed once, and resuspended at a density of 10^6 cells/mL in hT medium containing human IL-2 at 10 U/mL.
4. Day 7: 90% or more of the live cells are CD3-positive. The cells are ready for further manipulation. cell sorting, direct use in vitro, genomic DNA extraction, or infusion in vivo. Alternatively, when using selection strategies, drug-resistance selection can start on d 5.

3.2. Determination of Gene Transfer Efficiency

Direct methods are based on the accurate quantification of vector copy number integrated in the target cell genomic DNA. This result may be achieved
through a reliable and quantitative PCR-based assay or by quantitative Southern blot analysis (see Chapter 4).

Indirect methods are based on expression assays in single cells (see Section 1.3.). The most immediate assays are those in which the fraction of positive cells can be determined by screening or sorting based on marker detection: panning, column or magnetic separation, or FACS analysis. An example of such an analysis is shown in Fig. 1.

Absence of helper virus in viral stocks and in transduced target cell populations must always be verified (see Chapter 4).

### 4. Notes

1. When comparing infection efficiency achieved under different conditions, it is important to measure the infection efficiency at the same time-point after initiation of T-cell activation. Indeed, the percentage of positive cells may increase over time without necessarily reflecting an increased level of infection (e.g., if the noninfected cells have a shorter survival span than the infected cells).

2. The infection efficiency achieved by cocultivation of nonadherent target cells with the producers is often superior to infection using filtered viral stocks. Murine primary lymphocytes stringently require cocultivation with the packaging cell line. It is unknown whether cocultivation increases infection or survival of the target cells.

3. Gene transfer efficiency can be accurately assessed from expression studies if the following three conditions are met: all intact integrated vectors are expressed, all cells expressing the transgene are detected, and all positive cells bear one copy of the vector (the latter is a reasonable assumption when all cells are equally susceptible to infection and <50% of the cells are positive). After the prolonged culture necessary for drug-resistance selection, it is very difficult to estimate the fraction of cells that were originally transduced.

4. The procedures described herein result in infection rates of >10% in both CD4+ and CD8+ subsets, as determined by FACS analysis and confirmed by Southern blot analysis (Grallardo et al., manuscript in preparation). It is important to use high-titer viral stocks (>10^6 particles/mL).

### Acknowledgment

The author thanks V. Petronis for excellent assistance in the preparation of the manuscript.

### References


Methods for Retrovirus-Mediated Gene Transfer to CD34⁺-Enriched Cells

Alfred B. Bahnson, Maya Nimgaonkar, Edward D. Ball, and John A. Barranger

1. Introduction

Hematopoietic stem cells (HSC) provide for continuous replenishment of the entire immune and hematopoietic systems, and also replenish themselves in a process termed self-renewal (1). The HSCs can be enriched from hematopoietic tissues using MAbs that bind to the CD34 antigen, a universally recognized marker for hematopoietic progenitors (2–4). Enriched HSC populations are being widely investigated for use in transplantation and gene therapy because they appear to provide rapid hematopoietic reconstitution in myeloablated patients (5–11), and they offer good targets for gene transfer (12–17).

A major impediment to retroviral transduction of HSC has been their paucity in hematopoietic tissues. The CD34⁺ cells account for only 1–4% of mononuclear cells in the bone marrow (BM), <1% in peripheral blood (PB), and <1% in cord blood (CB) (18,19). CD34⁺ enrichment provides an important advantage for gene therapy protocols because it greatly reduces the quantities of viral supernatants needed to achieve a useful multiplicity of infection (MOI) compared with requirements for nonenriched cells from these sources. For example, with a titer of 10⁶ cfu/mL, full strength supernatant will achieve an MOI of 10:1 when cells are suspended at 10⁵ cells/mL. An average marrow infusion of 2 × 10⁸ nucleated cells/kg recipient body wt for a 70-kg patient would require suspension of the cells in 140 L of supernatant to achieve this MOI. Enrichment reduces these requirements by a factor of 100, making the procedure practical. By reducing associated quantities of cytokines and DMSO, costs and discomfort are also reduced.
Cytokines are required for in vitro survival and expansion of CD34+-enriched cells, but no known combination of cytokines can prevent the process of differentiation that accompanies in vitro handling (20–22). Cocultivation of CD34+ cells on adherent stromal cell layers appears to enable relatively long-term maintenance and, in some cases, low-level expansion of very early progenitors (23). Cycling among HSC is sought as a part of the transduction process with retroviral vectors, since cell division appears to be a requirement for retroviral transduction (24). Cycling may be brought about with cytokines applied in vitro (25,26) or with in vivo mobilization of cycling cells using cytokines and/or chemotherapy (27,28). An ideal prestimulation procedure would encourage the self-renewal of HSC without induction of differentiation.

Studies aimed at better defining factors and culture conditions for expansion of HSC may be crucial for successful application of gene therapy to hematopoietic disease. These efforts rely on assays for precursors and progenitor cells at various stages of differentiation. The colony forming methylcellulose assay provides an estimate of myeloid progenitor cells (CFU) (29), and may be combined with the assay for long-term culture initiating cells (LTC-IC) to measure more primitive hematopoietic progenitors (30). The severe combined immune deficient (SCID) mouse model permits assay of both myeloid and lymphoid cells, and is thus an assay for pluripotent stem cells (31).

Characterization of surface markers by flow cytometry provides more immediate information regarding quantities and phenotypes of subpopulations of hematopoietic cells. The CD34 antigen is highly expressed in early multipotential progenitors and colony forming cells (e.g., CFU-blast, pre-CFU, CFU-GEMM), whereas lower levels of CD34 expression are associated with more committed progenitors (e.g., BFU-E, CFU-GM, CFU-Meg, CFU-Eo, CFU-Osteoclast) (32). The CD34+ cells that are negative for CD38 and HLA-DR are considered to be among the earliest stem cells; they give rise to both hematopoietic and stromal elements (33). As these cells mature, they acquire a variety of surface antigens specific for their lineage of differentiation. For example, expression of the CD33, CD45, CD15, and CD11 antigens are associated differentially with myeloid and lymphoid differentiation. Differences in the proportions of the various subpopulations of CD34+ cells in BM compared with PB and G-CSF-mobilized PB may relate to the relative effectiveness of each of these sources for long-term engraftment and reconstitution in the patient (34,35). Ongoing studies of correlation between the assays and patient outcomes should eventually provide a useful definition of the stem cell phenotype.

Specific conditions for optimal retroviral transduction of CD34+-enriched cells are not conclusively determined. Reports and protocols differ with respect to many variables, e.g., the concentrations and combinations of cytokines used, timing of prestimulation and virus exposures, and the presence or absence of
Transfer to CD34+ Enriched Cells

stromal cells. The effectiveness of IL-3, IL-6, and SCF for promoting retroviral transduction and stem cell preservation has been demonstrated in murine (36–38) and human (14,39,40) cells. Cytotoxic prestimulation of stem cells in vivo with 5-fluorouracil (5-FU) is effective for enhancement of transduction efficiency in mice (36). Using an optimized protocol based on coculture with the murine producer cells, we and others have demonstrated transduction of cells capable of long-term reconstitution of lethally irradiated primary recipients, which yield progeny (self-renewal of stem cells) capable of reconstitution of secondary lethally irradiated recipient mice (41,42). Using an MFG-based vector, we have demonstrated expression in secondary recipients up to 12 mo posttransplant (41). These studies provide ample encouragement for the future potential of a well-optimized clinical protocol.

The effectiveness of 5-FU for enhancement of transduction of primate stem cells is less clear cut, however (43). Studies in nonhuman primates have yielded less efficient retroviral transduction and engraftment using protocols based on supernatant infection or coculture in comparison to the murine studies (44,45). Early results of human clinical trials using retroviral transduction of CD34+ or mononuclear hematopoietic cells are just beginning to be evaluated. The technique has been useful for demonstrating a contribution to relapse from the autologous bone marrow transplant (ABMT) in CML (46) and neuroblastoma (47). Low detection of transduced cells following gene therapy experiments in children with adenosine deaminase deficiency (48,49) and for marking ABMT cells in multiple myeloma and breast cancer patients (50), emphasizes a need for further optimization of protocols.

The comparison of results between the different studies using CD34+ cells is confounded by differing assay systems, differing vectors, differing supernatant titers, and many other variables. Estimates of transduction efficiency therefore range widely within and between different studies (Table 1).

Of the three protocols that have been approved by the Recombinant DNA Advisory Committee (RAC) for gene therapy of Gaucher disease, one (53) employs direct suspension of CD34+ cells into full strength vector-containing supernatant without prestimulation. The cytokines IL-3, IL-6, and SCF (concentrations were unspecified in the RAC proposal) and 4 mg/mL protamine sulfate are present, and the mixtures are completely changed daily for 3 d. Another (54) employs a 5-d incubation on a 14-d-old autologous long-term marrow stromal layer in a system containing 50% long-term culture medium (LTCM) and 50% vector-containing supernatant with IL-1, IL-3, IL-6, and SCF at 50 ng/mL and 5 mg/mL protamine sulfate. From the latter protocol, both nonadherent and adherent cells are harvested for assay and patient infusion. Problems may appear in evaluating the efficiency of gene transfer to HSCs because of transduction of adherent cells in this system. We reported an
<table>
<thead>
<tr>
<th>Source</th>
<th>Cytokines</th>
<th>Prestimulation</th>
<th>Infection</th>
<th>Assay</th>
<th>Efficiency, %</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM, PB</td>
<td>IL-3, IL-6, SCF, and combination</td>
<td>None; chemo</td>
<td>1 and 3 d</td>
<td>CFU-PCR</td>
<td>8, 25–33</td>
<td>14</td>
</tr>
<tr>
<td>BM</td>
<td>None</td>
<td>Chemo 1X</td>
<td>CFU-G418R</td>
<td>1–4</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>BM, PB</td>
<td>IL-3, IL-6, SCF</td>
<td>Chemo 3 d</td>
<td>CFU-G418R</td>
<td>2.5–40</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>CB</td>
<td>IL-3, IL-6, SCF</td>
<td>None 3 d</td>
<td>CFU-G418R</td>
<td>12–20</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>BM, PB</td>
<td>IL-3, IL-6, IL-11, SCF</td>
<td>None 4 d</td>
<td>CFU-PCR</td>
<td>25–42</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Gaucher</td>
<td>IL-3, IL-6, IL-11, SCF</td>
<td>None 4 d</td>
<td>LTC-IC</td>
<td>30–50</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>BM, PB</td>
<td>?</td>
<td>None 5 d</td>
<td>CFU-PCR</td>
<td>15–40</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>BM, PB</td>
<td>?</td>
<td>None 5 d</td>
<td>LTC-IC</td>
<td>14</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>BM (ADA)</td>
<td>?</td>
<td>None Coculture</td>
<td>CFU-Xyl-A</td>
<td>5–10</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>PB</td>
<td>Varies</td>
<td>Varies 1–3 d</td>
<td>Southern</td>
<td>10–30</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>BM, CB</td>
<td>IL-3, IL-6, SCF</td>
<td>1 d 1–4X</td>
<td>Southern</td>
<td>30–80</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>
Transfer to CD34+ Enriched Cells

experiment in which stromal Gaucher cells were transduced and nonadherent cells were not (16). In our clinical study (55), we propose to use a 1-d prestimulation of CD34+-enriched cells in LTMC media containing IL-3, IL-6, and SCF at 10 ng/mL followed by three, 12-h exposures to 50% fresh supernatant while maintaining cytokine concentrations at 10 ng/mL and protamine sulfate at 4 mg/mL.

It is clear that many problems remain to be solved in the use of CD34+ cells for gene therapy. Nevertheless, it is also clear from the numerous preclinical studies with human cells and with animal models that the potential for success is high. It is hoped that the methods described here will encourage others to contribute to this field.

2. Materials

2.1. Medium

1. Human long-term bone marrow culture medium (LTCM) is used for prestimulation and expansion of CD34+ cells and for long-term cultures. It consists of Iscove-Modified Dulbecco's Medium (IMDM) (Gibco-BRL, Gaithersburg, MD) containing 12.5% fetal bovine serum (FBS) (Hyclone, Logan, VT), 12.5% horse serum (HS) (Hyclone), 2 mM L-glutamine, 10^{-6}M 2-mercaptoethanol, 10^{-6}M alpha-thioglycerol, 1 µg/mL hydrocortisone, and penicillin/streptomycin

2. Packaging cells and producers are grown in 10% calf serum (CS) (Gibco-BRL) in Dulbecco’s Modified Eagle medium (DMEM) (Gibco-BRL) containing 50 U/mL penicillin, 50 µg/mL streptomycin, and 2 mM L-glutamine.

2.2. Additional Reagents

1. Cytokines, IL-3, IL-6, and stem cell factor (SCF) are prepared as concentrated stock solutions in LTCM. The 1000X contains 10 µg/mL of each cytokine, and a combination 100X stock solution containing all three cytokines at 1 µg/mL each is used for addition of factors to small volumes of medium or supernatant. Aliquots are stored at -80°C until needed.

2. Protamine sulfate is prepared as a concentrated stock solution in phosphate buffered saline (PBS); 1000X contains 4 mg/mL.

3. Methylcellulose medium (without erythropoietin) is obtained ready-to-use from Stem Cell Technologies, Vancouver, BC.

4. CFU-GM colony DNA extraction mixture consists of 1.5 parts glycogen, 10 parts 2M sodium acetate at pH 4.5, 100 parts phenol, and 20 parts chloroform. Homogenize immediately before use by vigorous shaking.

2.3. Immunoaffinity Column and Anti-CD34 Antibody

The biotinylated anti-CD34 antibody, the avidin column, and necessary reagents (CEPRATE LC [CD34] Cell Separation System) are supplied by CellPro, Bothell, WA.
3. Methods

3.1. Generation of Amphotropic Vector-Containing Supernatants

Amphotropic producer cell lines containing nonselectable markers may be generated by infection with filtered supernatants from ecotropic producers, stably or transiently transfected with the vectors in plasmid form. The amphotropic packaging cells are infected multiple times if necessary to obtain clones which, on the average, carry several copies of the vector of interest. The highest titer clones are used to produce stock supernatants.

1. Obtain ecotropic supernatant from stably or transiently transfected ecotropic producer cells
2. Split freshly confluent amphotropic producer cells 1:40 one day prior to infection.
3. The next day, remove the medium from the amphotropic packaging cells and apply a 1:2 dilution of ecotropic supernatant in fresh medium containing polybrene (8 μg/mL final concentration), and incubate for 12 h or overnight.
4. Repeat step 3 one or more times
5. Clone infected cells by limiting dilution in a 96-well plate or by seeding at low density in a tissue culture dish.
6. Transfer clones to 24-well plates and harvest supernatants as cells reach confluence
7. Screen supernatants for virus activity by infecting appropriate targets cells, e.g., 3T3, and assay for expression of the vector of interest.
8. Select highest titer clone and expand into 15-cm tissue culture dishes.
9. Replace the medium daily as the cells become confluent. Filter the supernatants and store at -70°C.
10. After the cells become fully confluent, do not split them but continue to feed once or twice daily as long as desired (see Note 1), filtering and freezing each collection. With each collection, aliquot a small volume into a cryotube.
11. Following a series of collections, assay and compare the small aliquots to evaluate conditions for, and collections having, the highest titer.

3.2. Enrichment of CD34+ Cells

Bone marrow cells from normal patients are obtained from allogeneic transplant donors, cord blood cells are obtained from discarded placentas following normal delivery, and G-CSF-primed peripheral blood cells are obtained from patients undergoing autologous bone marrow transplant combined with high-dose chemotherapy. The CD34+ enrichment is performed using an MAb and an avidin-biotin immunoaffinity system according to the manufacturer’s instructions.

1. Hematopoietic cells are applied to a Ficoll-hypaque gradient, and the light density cells are washed and resuspended at 1–2 × 10^6/mL in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA)
Transfer to CD34+ Enriched Cells

2. Biotinylated anti-CD34 MAb (80 μL/mL) is added and cells are incubated at room temperature for 25 min.
3. The antibody-labeled cells are applied to the prepared avidin column, followed by a wash with PBS containing 1% BSA.
4. Adsorbed CD34+ cells are released by mechanical manipulation.
5. Samples may be analyzed with a direct staining method on a FACScan flow cytometer (Becton Dickinson, Rutherford, NJ) as described elsewhere (17).

3.3. Prestimulation and Transduction of Human CD34+ Cells

The transduction protocol consists of two phases: a 24-h prestimulation step in LTCM containing IL-3, IL-6, and SCF (10 ng/mL each) followed by repeated additions of vector-containing supernatants over a period of 2–3 d. A multiplicity of infection (MOI = infectious vector copy:3T3 target cell ratio) of 10–20, and a cell concentration of ≤5 × 10⁵/mL are maintained (see Note 2). In each experiment, nontransduced control cells are subjected to mock transduction or transduction with a different vector in parallel with cells receiving the vector of interest.

1. Count the CD34+-enriched cells and suspend them at 2.5 × 10⁵/mL in LTCM containing IL-3, IL-6, and SCF at 10 ng/mL. The total number of cells obtained will determine the volume and type of vessel needed to accommodate them. We sometimes begin with as few as 2.5 × 10⁵ cells in 1 mL in a 24-well plate. Incubate the cells for 24 h.
2. The following day, add an equal volume of virus-containing supernatant to which has been added 8 μg/mL of protamine sulfate and 10 ng/mL of IL-3, IL-6, and SCF. Incubate 12–24 h.
3. For the next infection, reduce the volume by drawing off half the medium or transfer the cells to a larger plate if necessary and add an equal volume of freshly thawed virus-containing supernatant containing 4 μg/mL protamine sulfate and 10 ng/mL of the three cytokines. Incubate for 12–24 h.
4. Repeat step 3 with another aliquot of freshly thawed virus-containing supernatant.
5. After the final infection, thoroughly resuspend the samples and determine the cell concentrations.
6. Plate 10⁴/mL or fewer cells in methylcellulose cultures according to the instructions of the supplier (Stem Cell Technologies, Vancouver, Canada). These are assayed after 12–14 d by PCR amplification (see Section 3.4.1.) of individual colonies to yield an estimate of the efficiency of transduction of CFU-GM.
7. Cells may be plated into irradiated long-term bone marrow cultures at this time (see Section 3.4.3.).
8. Expand the remaining cells by addition of LTCM containing IL-3, IL-6, and SCF or other cytokine combinations, if desired. Maintain cell concentrations below 10⁶/mL. The CD34+-enriched cells will continue to differentiate and expand between 2–3 logs for approx 3 wk. Harvest cells for assay of gene product or for Southern blot hybridization analysis, as appropriate (see Note 3).
3.4. Determination of Transduction Efficiency

3.4.1. Extraction of DNA from CFU-GM Colonies for PCR Analysis

The polymerase chain reaction is performed on individual methocel colonies following phenol/chloroform microextraction of the DNA as described by Deisseroth et al. (46, see Note 4).

1. Select isolated colonies using a micropipet and a steady hand while observing on an inverted microscope. Transfer the colonies to Eppendorf tubes.
2. Add 100 µL of homogenized extraction mixture, vortex, and allow to sit on ice for 15 min.
3. Centrifuge the samples for 10 min at 10,000g.
4. Transfer the top aqueous layer to a new Eppendorf tube and add 100 µL of isopropanol.
5. Store the samples at -20°C for 1 h or overnight, then centrifuge for 10 min at 10,000g.
6. Remove the supernatant, wash the pellet with 70% ethanol, centrifuge, remove the wash, and allow the pellet to air dry.
7. Redissolve the pellet in 20 µL water for use in a standard PCR reaction.

3.4.2. Determination of Copy Number by Southern Blot

For determination of copy number by Southern blot hybridization, infected and control sample bands on autoradiographs are compared with bands produced using spiked amounts of DNA from cell lines carrying known vector copy numbers per cell.

1. Infect a human or mouse cell line with vector-containing supernatants. If the titer is high (e.g., 10⁷ or greater) a single infection may transduce the majority of the target cells with one or more copies per cell. For lower titer supernatants, multiple infections may be necessary to permit isolation of a number of infected clones.
2. Clone the infected cells by limiting dilution, and expand the clones to obtain at least 100 µg of DNA. Usually, 10⁷ cells is sufficient. Be sure to cryogenically preserve viable samples of each clone.
3. Digest the DNA from each clone with an enzyme that cuts only once within the vector, outside of the region used for the probe. By this means, each different integration site will yield a differently sized DNA fragment, depending on the distance from the recognition sequence within the vector through the probed region to the nearest recognition site in the flanking genomic DNA.
4. Also, digest another portion of DNA with an enzyme that cuts only within the LTRs, thereby yielding a single band of defined mol wt, independently of the number of different integration sites.
5. Run the digests on an agarose gel, blot, and probe the blot by standard methods (e.g., see ref. 56).
6. Select several clones that reveal one or more copies of the vector. Confirm the clonality by subcloning and reanalysis of each subclone. Confirm the copy num-
ber by using a second unique enzyme to give the same number of bands that were obtained from step 3.

7. Use the DNA obtained from the clones to spike into noninfected control DNA in order to generate standard mixtures that are equivalent to the desired copy numbers or fractions of copies per cell (see Note 5).

8. Digest these standard mixtures along with unknown samples using an enzyme that recognizes a site within the LTRs.

9. Run the samples and standards together in an agarose gel, blot, and probe.

10. Using a beta scanner, densitometer, or visual estimation, compare vector band intensities of the known copy number standards with the samples. Ideally, prepare a standard curve that encompasses the sample band intensities.

11. Use the standard curve or extrapolate to determine sample copy numbers based on the standard mixtures.

3.4.3. Long-Term Bone Marrow Culture (LTC) Initiating Cells

Southern analysis of in vitro expanded cells and PCR analysis of CFU-GM provide estimates for transduction efficiency, but neither of these methods yields conclusive evidence for transduction of primitive stem cells. Initiation of hematopoiesis in irradiated long-term cultures, and subsequent demonstration of transduced CFU-GM from such cultures after 5 wk, is indication that progenitors more primitive than CFU-GM have been transduced.

1. At least 2 wk prior to transduction of CD34+ -enriched samples, establish LTC stromal layers by charging T25 tissue culture flasks with 2.5 × 10^7 mononuclear cells from human bone marrow in 10 mL of LTCM.

2. Incubate the flasks at 37°C for 3 d and then at 33°C thereafter. Feed the flasks weekly by replacement of half the medium with fresh LTCM.

3. Prior to seeding of cells, expose the stroma to 2000 rad. Change the medium and incubate overnight.

4. Seed transduced and control CD34+ -enriched cells at a concentration of 5 × 10^5 cells per flask.

5. Continue to incubate the flasks at 33°C with weekly replacement of half the medium.

6. After 6 wk, plate the adherent and nonadherent cells in methocel cultures for generation of colonies from CFU-GM, and analyze for presence of the vector sequence by PCR.

4. Notes

1. Optimum conditions for collection of supernatant may vary between different packaging lines. With YCRIP producer lines, we have found that the highest titer supernatants are obtained after the cells reach confluence. As the dishes become confluent, feed the cells fresh medium daily to maintain a healthy monolayer, but do not split the cells. Instead, collect overnight supernatants from dishes (15 cm) fed with 30 mL of medium the previous afternoon, and collect 8-h supernatants during the afternoon from dishes fed with 10 mL of fresh medium in the morning.
The transduction process is under continuing investigation and is complicated by many variables. It is often difficult to obtain the quantity of cells needed to make multiparameter comparisons in a single experiment, and unknown variables between experiments further complicate this objective. Unexpected loss of titer is sometimes observed. Variables that we have investigated include the requirement for cytokine-prestimulation (24 h seems to be a requirement), specific requirement for SCF in addition to IL-3 and IL-6 (SCF adds an increment of improvement), the presence of fibroblasts (not apparently helpful), use of centrifugation to enhance transduction (can definitely help, see ref. 57), multiple infections (definitely additive up to 3-4X, see ref. 16), and effect of cryogenic preservation of cells (does not prevent subsequent transduction after thawing).

The protocol we use maintains a constant virus concentration at the initiation of each infection cycle, i.e., a 1:1 mixture of fresh supernatant in medium is applied. We find virus concentration (dilution ratio) to be a reproducibly important variable for infection of 3T3 targets, although the effect is not linear, higher transduction occurs at higher supernatant concentrations. Supernatant titer appears to be of crucial importance, and every effort should be put into obtaining and preserving high titer supernatant stocks.

Since these cells undergo 2-3 log expansions during this time, the copy number measurement represents an overall average for the progeny of transduced progenitors, and should theoretically be comparable to transduction efficiency as measured by PCR of CFU-GM. It is as if all of the colonies and any background cells were pooled and assayed as an average. It should be kept in mind that background cells, i.e., cells that do not proliferate into colonies but which survive or may otherwise contaminate CFU-GM colonies with DNA, may contribute to positive PCR results. Controls for highly transduced samples should therefore include analyses of random samples of methocel taken from areas between visible colonies. Positive results for such control samples indicate a likelihood for overestimation of transduction efficiency based on CFU-GM.

Protocols for direct PCR analysis of boiled, proteinase K-treated methocel colonies were attempted prior to the use of the more involved phenol/chloroform extraction procedure. The improvement in signal sensitivity with the procedure reported by Deisseroth et al. (46) has been well worth the effort.

In spiking human DNA with infected control DNA, correction is made for the difference between the mouse and human genome size, i.e., 2.9 and 3.7 x 10^9 bp per haploid genome, respectively. Thus, 78% as much mouse DNA is added to spike a given quantity of human DNA in comparison to the amount that would be added to spike the same quantity of mouse DNA at the same copy number. For example, if a characterized murine clone contains 5 copies per cell, then 2 µg should be spiked into 8 µg of control mouse DNA for a 1 copy standard, but only 1.6 µg should be spiked into 10 µg of control human DNA for a 1 copy standard. In each case, the total DNA for the species is 10 µg, and quantitation of the vector copy number is dependent on the precision of this loading in each lane for unknown samples as well as on the precision of the standard DNA spike loading.
Transfer to CD34+ Enriched Cells

If endogenous sequences are present that hybridize to the probe or to a second probe without interference with the vector sequence, then these bands may be used as reference bands to normalize each lane for variations in the quantity of DNA loaded. In this case, the precision of the loading of control noninfected DNA in the spiked samples is also important. The latter potential source of error could be avoided in the ideal case in which several clones were available carrying a range of copy numbers per cell for the species of interest. Consideration should also be given to the karyotype of the infected cell lines being used for copy number standards. If a cross-hybridizing reference band is used to normalize DNA quantity in each lane, then it is important that the chromosomes that carry the relevant cross-hybridizing sequence(s) are present in normal diploid amount or that the sex be ascertained if the sequence is sex-linked. It is also advantageous to use euploid lines, if possible, since significant aneuploidy requires complicated adjustments to be made in comparing control DNA to infected primary cells from chromosomally normal individuals. All of these factors contribute to the uncertainty of the Southern technique for quantitation, but to the extent to which they may be controlled, it seems proper to do so.

References


Methods for the Use of Genetically Modified Keratinocytes in Gene Therapy

Sabine A. Eming and Jeffrey R. Morgan

1. Introduction

Recent advances in molecular genetics have resulted in the development of new technologies for the introduction and expression of genes in human somatic cells. Recombinant retroviral vectors have become a popular method for introducing such genes, primarily because the frequency of gene transfer is usually high and the introduced genes are stably integrated into the host genome. Gene therapy and gene transfer technology, although typically thought of as a means to correct genetic disease, have other potential clinical applications in the areas of protein delivery and tissue engineering. Numerous types of cells/tissues have been successfully genetically modified and are potential targets for retroviral mediated gene therapy (1). The successful use of these tissues in gene therapy relies on methods to cultivate and transplant the cells after genetic modification.

In this chapter, we describe the use of retrovirus-mediated gene transfer to genetically modify human keratinocytes, the principal cell type of the epidermis. Epidermal keratinocytes are an attractive target for gene therapy because they are easy to obtain, grow rapidly under appropriate culture conditions, and can be transplanted. Using appropriate culture conditions, the lifetime of diploid human keratinocytes in vitro has been reported to be 50–140 population doublings (2). In fact, the keratinocytes from the epidermis of a 1-cm² biopsy can be expanded to an area of 1 m² in approx 30 d in vitro (3). When grown to confluence, cultured keratinocytes form a coherent multicell, layered epithelial sheet, that organizes itself into a differentiated epidermis when grafted to animals or humans (4–6). The successful use of cultured epidermal sheet grafts
as long-term wound coverage indicates that the epidermal stem cell survives in culture and can reconstitute a differentiated and renewable epidermis.

Although the primary role of the mammalian epidermis is the formation of a protective barrier between the individual and the external environment, recent work has shown that keratinocytes synthesize and secrete numerous proteins that are involved in a variety of metabolic activities; e.g., keratinocytes are a source of many cytokines (7) It has been shown that at least two proteins (apolipoprotein E, type VII collagen of dermal anchoring fibrils) endogenously secreted by keratinocytes can cross the basement membrane separating the epidermis from the dermis (8,9). This observation suggests that epidermal-derived proteins, in addition to acting at local sites, may affect distant cells, tissues, and organs.

Cultured primary keratinocytes have been successfully genetically modified with retroviral vectors encoding several different genes, including human growth hormone (hGH) (10), neomycin (neo) (11), the β-subunit of human chorionic gonadotrophin (β-hCG) (12), clotting factor IX (13), and platelet-derived growth factor (PDGF-A) (14). When grafted as an epithelial sheet onto athymic mice, modified keratinocytes reconstituted a stratified epithelium with well-defined stratum basale, spinosum, granulosum, and corneum.

In addition to the delivery of therapeutic proteins, genetically modified skin grafts potentially can be used toward the correction of inherited diseases of the skin. Much progress has been made in identifying the molecular defects in several inherited diseases of the skin, including xeroderma pigmentosum and various forms of epidermolysis bullosa, a family of skin-blasting diseases (15,16). Grafting of autologous keratinocytes, modified to express a functional copy of the defective gene, could potentially restore normal epidermal function at those sites of the body most severely affected by disease.

In this chapter, we describe methods for the culture of diploid human keratinocytes, the genetic modification of these cells by retrovirus-mediated gene transfer, and the transplantation of modified keratinocytes to the athymic mouse.

2. Materials

2.1. Cell Culture and Genetic Modification

2.1.1. Fibroblast Feeder Layer/Virus-Producing Cell Line

1. Fibroblast feeder layer: 3T3-J2 mouse fibroblast cell line (originally provided by H. Green, Department of Physiology and Biophysics, Harvard Medical School, Boston, MA)

2. Virus-producing cell line: Ψ-CRIP amphotropic packaging cell line, a derivative of NIH 3T3 fibroblasts that expresses retroviral gag, pol, and env gene products (originally obtained from R. Mulligan, Whitehead Institute of Biomedical Research, Cambridge, MA).
Keratinocytes in Gene Therapy

3. Fibroblast tissue-culture medium: Dulbecco’s Modified Eagle’s Medium (DMEM, high glucose, L-glutamine, 110 mg/L sodium pyruvate, Gibco-BRL, Gaithersburg, MD), bovine calf serum 10% (HyClone, Logan, UT), penicillin-streptomycin (Boehringer, Indianapolis, IN) 100 IU/mL - 100 μg/mL.

4. Mitomycin C (Boehringer): 15 μg/mL in serum-free DMEM.

2.1.2. Keratinocytes

1. Source for human keratinocytes: newborn foreskins (average size: 1–2 cm²).

2. KCM (keratinocyte tissue-culture medium):
   a. DMEM/Ham’s F12 medium (Gibco-BRL) (3:1).
   b. Fetal bovine serum 10% (JRH Bioscience, Lenexa, KS).
   c. Penicillin-streptomycin 100 IU/mL - 100 μg/mL.
   d. Adenine (6-aminopurine hydrochloride, Sigma, St. Louis, MO); make up fresh at time medium is prepared; prepare stock (50X) of 1.2 mg/mL in DMEM/F12 (3:1), adjust pH to 7.5 with 1N NaOH, sterilize by filtration using a 0.45-μm filter; add 2 mL of stock to 100 mL of KCM; final concentration: 1.8 × 10⁻⁶ M.
   e. Cholera toxin (Vibrio cholerae, Type Inaba 569 B, Calbiochem, La Jolla, CA); prepare concentrated stock of 10⁻⁵ M in distilled water, store at 4°C; take 0.1 mL of concentrated stock and make up to 10 mL with DMEM (10% fetal calf serum), sterilize by filtration using a 0.45-μm filter and aliquot in 1-mL portions (10⁻⁷ M), store at -20°C; add 0.1 mL to 100 mL of KCM; final concentration: 10⁻¹⁰ M.
   f. Epidermal growth factor (mouse, Collaborative Biomedical Products, Bedford, MA); resuspend lyophilized material in distilled water to prepare stock of 10 μg/mL; sterilize by filtration using a 0.45-μm filter and aliquot in 1-mL portions, store at -20°C; add 0.1 mL to 100 mL KCM with first medium change; final concentration: 10 ng/mL.
   g. Hydrocortisone (chromatographic standard, Calbiochem); prepare stock of 5 mg/mL in 95% ethanol, store at 4°C; take 0.4 mL of stock and make up to 10 mL with serum-free DMEM, sterilize by filtration using a 0.45-μm filter, and aliquot in 1-mL portions; store at -20°C; add 0.2 mL to 100 mL of KCM; final concentration: 0.4 μg/mL.
   h. Insulin (pork, 100 U/mL [3.8 mg/mL], Novo Nordisk, Danbury, CT); add 0.13 mL of stock to 100 mL of KCM; final concentration: 5 μg/mL.
   i. T/T3 stock (transferrin/triiodo-L-thyronine stock); T stock (transferrin, human, partially iron saturated, Boehringer) 5 mg/mL in PBS; T3 stock (3,3',5 triiodo-L-thyronine, sodium salt, Sigma) dissolve 13.6 mg in the minimum amount of 0.02N NaOH and make volume up to 100 mL with distilled water, sterilize by filtration using a 0.45-μm filter and store at -20°C (2 × 10⁻⁴ M); add 0.1 mL T3 stock to 9.9 mL of T stock, sterilize by filtration using a 0.45-μm filter and aliquot in 1-mL portions, store at -20°C; add 0.1 mL of T/T3 stock to 100 mL of KCM; final concentration transferrin: 5 μg/mL, triiodo-L-thyronine. 2 × 10⁻⁶ M.
3. KCM selective growth medium: If KCM is used as selective growth medium for cells carrying the drug resistance gene neo (neomycin phosphotransferase), add 0.6 mg/mL G418.

4. PBS (phosphate-buffered saline). 138 mM NaCl, 2.7M KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄; sterilize by filtration using a 0.45-µm filter.

5. EDTA solution ([ethylenedinitriol]tetraacetic acid disodium salt, Boehringer) 5 mM in PBS; sterilize by filtration using a 0.45-µm filter.

6. Trypsin solution (trypsin 1-300, ICN Biochemicals, High Wycombe, UK): D-glucose 0.1% (w/v), trypsin 0.1% (w/v) in PBS, pH 7.5; sterilize by filtration using a 0.45-µm filter, store at -20°C, avoid repeated thawing/freezing.

7. Trypsinizing flask (25 mL, Wheaton Scientific, Millville, NJ)

8. Rhodamine B (Sigma): 1% (w/v) in distilled water.

9. Formaldehyde (Sigma, 37%): dilute stock 1:10 (v/v) in PBS

2.2. Grafting of Modified Cells

1. Dispase II (Boehringer): 2.4 mg/mL in serum-free DMEM; prepare fresh before use.

2. Ethanol: 70% in distilled water.

3. Anesthesia (2,2,2,-tribromoethanol, Aldrich, Milwaukee, WI): prepare concentrated stock of 1.6 g/mL in 2-methyl-2-butanol alcohol; store at 4°C. Before use, prepare working solution by diluting 12-µL (concentrated stock)/mL 0.9% NaCl at 40°C, sterilize by filtration using a 0.45-µm filter.

4. Surgical instruments: straight, sharp scissors, curved scissors, forceps.

5. Ligaclips (LC-100, small, Ethicon, Somerville, NJ).


7. Ligaclip applier (LC-105, small, Ethicon).


9. Silastic-sheeting (0.005 x 6 x 8 in., Dow Corning, Midland, MI): Wash off powder as recommended by the manufacturer, prepare rectangular sheets 1.8 x 1.8 cm (slightly larger than graft) and sterilize by autoclaving.

10. Dental wax, byte ryte, (Mizzy, Cherry Hill, NJ).

11. Tris-buffer: 25 mM Tris-HCl, 1 mM EDTA, pH 7.5.

3. Methods

3.1. Keratinocyte Cell Culture

Although there are several methods for the culture of human keratinocytes, the method developed by Rheinwald and Green is preferred for two reasons: the cells have great growth potential and the epithelia produced from these cultures have had the most clinical application. In addition to media containing fetal calf serum and added factors (i.e., EGF, cholera toxin, transferrin, insulin, hydrocortisone), this method uses a fibroblast feeder layer that is crucial to the clonal growth of keratinocytes (17). The fibroblast feeder layer also conditions the media and inhibits the growth of contaminating dermal fibroblasts present
in the biopsy used to initiate primary cultures. As the cells reach confluence, they stratify and form multicell, layered sheets that can be detached from the dish as an intact sheet and grafted. We have modified the Rheinwald and Green method to maintain these conditions for optimal cell growth and also for the genetic modification of the cells by retrovirus-mediated gene transfer.

3.1.1. Fibroblast Feeder Layer

Cocultivation of the epidermal cells with a feeder layer is critical for the clonal growth of keratinocytes and optimal growth is important for retrovirus-mediated gene transfer.

1. Grow 3T3-J2 cells in 75-cm² flasks or other suitable dishes.
2. Before the primary keratinocytes from the processed biopsy can be added, the feeder layer must be prepared by treating the fibroblasts with mitomycin C, which limits the further proliferation of the fibroblasts. Moreover, as shown by Rheinwald and Green, the density of the feeder cells is important for optimal keratinocyte growth. Prepare feeder layer by growing the cells to 50% confluence and incubating cells for 2 h at 37°C with 15 mL of DMEM supplemented with mitomycin C; remove mitomycin C medium and wash cells twice with DMEM Alternatively, grow the 3T3-J2 cells to near confluence, treat the cells with mitomycin C, trypsinize, and inoculate 2 × 10⁶ cells /75-cm² flask (use comparable densities for other size dishes) in fibroblast culture media. Let the cells attach overnight (37°C, 10% CO₂) before adding keratinocytes.
3. Two hours prior to adding keratinocytes, replace fibroblast media with 12 mL KCM (without EGF) and equilibrate flasks in humidified incubator (37°C, 10% CO₂)

3.1.2. Preparation of Primary Cultures of Keratinocytes from Biopsy Material

1. Place each biopsy in individual 15-mL sterile culture tubes containing KCM (without EGF) at 4°C. Keep biopsies on ice until processed.
2. Biopsies should be processed no later than 6–8 h after they were obtained. Place the biopsy in a 10-cm dish containing 10 mL PBS. If necessary, dissect to flatten the tissue. Trim off as much connective tissue as possible using the curved scissors.
3. Transfer the biopsy to a 50-cm² culture tube containing 10 mL PBS; wash the biopsy by vigorously shaking the tube for approx 60 s. Repeat this washing step 10 times by transferring the biopsy to a new culture tube containing fresh PBS.
4. Place the tissue into a 10-cm dish and, with curved scissors, carefully remove any remaining connective tissue from the dermis. Transfer the epithelium into a 60-mm dish, add 3 mL trypsin solution, and mince very finely (tissue fragments of approx 0.5 mm³) with sharp scissors. Transfer the minced tissue suspension into a trypsinizing flask. Prewet the spout of the flask with EDTA solution to avoid sticking of the tissue to the glass. Rinse culture dish by adding 3 mL EDTA solution and transfer to the flask. Add 3 mL of trypsin and 3 mL of EDTA solutions (prewarmed) to the flask. Gently stir the tissue fragments at 37°C.
5. After 60 min, remove the flask from the incubator, allow the large tissue fragments to settle, and pour off the cell suspension into a 15-mL culture tube. Add fresh trypsin/EDTA solution (12 mL of trypsin/EDTA solution 1:1 at 37°C) and repeat trypsinization step for one more harvest. Pellet the cells obtained from the first harvest by centrifugation at 70g for 5 min. Resuspend cell pellet in 3 mL KCM (without EGF) and add the cells from one harvest (1–2 × 10^6 cells) to a 75-cm² flask containing mitomycin C treated 3T3-J2 cells and 12 mL KCM. Each harvest should be plated into a separate flask. To get even seeding of the cells, the incubator shelf should be leveled and the flasks should not be stacked for the first 24 h.

6. Refeed cultures with 15 mL KCM every 4 d, adding EGF at the first medium change after inoculation. When the culture becomes dense, use 18 mL/75-cm² flask.

7. Primary cultures inoculated with epidermal cells from one harvest should approach confluence within 12–14 d and will contain 12–15 × 10^6 keratinocytes/75-cm² flask. The cultures can now be trypsinized and subcultured. As keratinocytes grow into a confluent epithelium, most of the feeder layer is displaced. Prior to trypsinization, remove residual fibroblasts with a brief wash with EDTA solution as follows: Add 10 mL EDTA solution/75-cm² flask, incubate for 1 min at 37°C, shake the flask to remove fibroblasts, and aspirate off the EDTA solution; repeat washing step if needed. Next, trypsinize the keratinocytes by incubation with 5 mL trypsin solution and 5 mL EDTA solution/75-cm² flask for 15–20 min at 37°C. Neutralize the trypsin/EDTA solution by adding 10 mL KCM/75-cm² flask; pipet the cell suspension repeatedly to dissociate residual cell aggregates. Inspect under the microscope to be sure that trypsinization is complete. Pellet the cells by centrifugation at 70g for 5 min, resuspend cell pellet in the appropriate volume of KCM (without EGF) to obtain the desired cell density and pass cells to a mitomycin C-treated feeder layer.

3.1.3. Colony Forming Efficiency

The colony forming efficiency of normal and genetically modified keratinocytes is a good measure of the growth performance of the cells and should be performed at each subculture.

1. At each subculture, inoculate 100 keratinocytes (modified or unmodified cells) in triplicate 60-mm dishes containing 5 × 10^5 mitomycin C-treated 3T3-J2 cells.
2. After 12 d, fix the colonies with buffered formaldehyde (10 min), then stain with rhodamine B (30 min). Vigorously growing colonies should form uniform, circular colonies with a plating efficiency of 20–30%. Irregular-shaped colonies are more differentiated and have diminished growth potential.

3.2. Retroviral Mediated Gene Transfer into Keratinocytes

Recombinant retroviruses provide an effective means for the efficient and stable introduction of a gene into a dividing cell population. The details of the molecular biology of retrovirus-mediated gene transfer is beyond the scope of
this chapter and the reader is referred to several review articles (18–20). Never-
theless, all recombinant retroviruses are produced by specialized packaging
cell lines that are a derivative of 3T3 fibroblasts. Several groups have made
comparable packaging cell lines and these packaging cell lines express the viral
gag, pol, and env genes necessary to package recombinant retroviruses (21).
Moreover, packaging cell lines can be easily cultured and they continuously
shed recombinant retrovirus into the media. The recombinant retroviruses pro-
duced by these packaging cell lines are free of wild-type replication virus but
are able to integrate and express the gene(s) of interest in a target cell.

3.2.1. Use of Virus-Producing Cell Line as Feeder Layer

1. To genetically modify human keratinocytes, the 3T3-J2 fibroblasts feeder layer
   is replaced with a packaging cell line that produces the recombinant retrovirus of
   interest. The keratinocytes are cocultivated with the virus-producing cell line that
   sheds recombinant virus into the media that infects the growing keratinocytes.

2. To prepare the virus-producing cell line for keratinocyte cocultivation, handle
   the cells as the 3T3-J2 cell line described in Section 3.1.1. One day prior to moc-
   culation with keratinocytes, treat virus-producing cells with mitomycin C,
   trypsinize, and plate 2 x 10^6 cells/75-cm^2 flask. For control cultures (unmodified
   keratinocytes), prepare parallel flasks with 3T3-J2 fibroblasts.

   The cocultivation of keratinocytes with the virus-producing cell line pro-
   vides not only a feeder layer for proper growth of keratinocytes but also the
   continuous production of recombinant retrovirus that integrates into the divid-
   ing keratinocytes.

3.2.2. Infection of Keratinocytes

1. After about 12 d, the primary cultures of keratinocytes are ready to be passed
   Dissociate the cells by trypsin treatment and inoculate 10^6 keratinocytes/75-cm^2
   flask containing 2 x 10^5 virus-producing cells (for unmodified controls, use
   3T3-J2 cells).

2. After 4–6 d of cocultivation with the virus-producing cells, the cells have been
genetically modified and the keratinocytes are ready to be subcultured. Prepare
parallel cultures for grafting, protein analysis, DNA/RNA isolation, and plating
efficiency analysis. An overview for the preparation of tertiary cultures is given
in Table 1 and will vary depending on the experiments needs.

3.2.2.1. TIME-COURSE OF PROTEIN SECRETION
   BY GENETICALLY MODIFIED KERATINOCYTES

   If the transferred gene encodes a secreted protein, its synthesis can be moni-
tored by analyzing conditioned culture media for the presence of the protein.

1. Inoculate 10^6 modified or unmodified keratinocytes/10-cm dish, containing 10^6
   mitomycin C-treated 3T3-J2 cells; prepare two dishes for each condition.
Table 1
Preparation of Tertiary Cultures

<table>
<thead>
<tr>
<th>Culture (dish size)</th>
<th>Number of dishes per condition(^a)</th>
<th>3T3-J2 (cells/dish)</th>
<th>Keratinocytes (cells/dish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein analysis (10 cm)</td>
<td>2</td>
<td>10^6</td>
<td>10^6</td>
</tr>
<tr>
<td>Plating efficiency (60 cm)</td>
<td>3</td>
<td>5 \times 10^5</td>
<td>100</td>
</tr>
<tr>
<td>Graft (35 mm)</td>
<td>as needed(^b)</td>
<td>2 \times 10^5</td>
<td>10^5</td>
</tr>
</tbody>
</table>

\(^a\)Condition, modified or unmodified keratinocytes.
\(^b\)35-mm dish = 1 graft, 1 graft/mouse.

2. After cells reach confluence (6–7 d), wash cells once with DMEM and add 30 mL of KCM (with EGF)/10-cm dish. Over a 4-d period, take two time points (AM/PM, 1 mL each) per day. Pellet the cell debris by brief centrifugation at 15,000g; store aliquots at −20°C.

3. At the end of the time-course, trypsinize and count keratinocytes. Normalize protein production to cell number.

3.2.2.2. Efficiency of Gene Transfer

Several methods can be used to determine the efficiency of gene transfer. If the vector contains a drug resistance gene, infected cells can be selectively grown in culture medium containing the corresponding drug. However, the feeder layer used to grow the keratinocytes in selective media must be resistant to the drug. The following example is for vectors encoding the drug resistance gene neo.

1. Trypsinize modified keratinocytes as before.

2. Pass cells onto dishes containing a modified 3T3-J2 feeder layer. These modified 3T3-J2 feeder cells have been previously genetically modified to express the neo gene and are resistant to the antibiotic, G418.

3. Culture keratinocytes on the modified feeder layer in KCM containing G418. After about 6 d, drug-resistant keratinocyte colonies will form and can be expanded in number.

Alternatively, the efficiency of gene transfer can be assayed by estimating the number of gene copies of the virus integrated into the cellular DNA of the epidermal cells. Total genomic DNA can be isolated from modified keratinocytes at the end of the time-course (described in Section 3.2.2.1.).

1. After trypsinization and counting, wash cells with PBS.

2. Centrifuge cells at 120g for 10 min, discard supernatant, and store cells at −80°C until processing for DNA.
3. Prepare genomic DNA using any of the standard protocols to prepare high-mol-wt DNA from mammalian cells.

4. DNA is digested with a restriction enzyme that cuts once in each LTR of the retroviral genome. After fractionation by agarose gel electrophoresis, the DNA is transferred to a membrane and the number of retrovirus gene copies is estimated by Southern blot hybridization with a labeled probe specific for the transferred gene.

3.2.2.3. Preparation of Modified Grafts

1. Inoculate 10^5 modified or unmodified keratinocytes into 35-mm dishes, containing 2 x 10^5 mitomycin C-treated 3T3-J2 cells.

2. Within 6–7 d, these cultures reach confluence. Graft cultures no more than 2 d later. Overconfluent cultures form blisters and should not be grafted.

3.3. Grafting of Modified Epithelial Sheets

3.3.1. Dispase Treatment

1. When the epithelium reaches confluence, wash cultures twice with serum-free DMEM and add 4 mL of dispase solution. After 30 min at 37°C, the edges of the epithelium will loosen and half of the medium should be removed to minimize folding and curling of the graft. Within 1 h at 37°C, the epithelium should completely detach from the plastic dish and float in the medium.

2. Wash epithelium several times with DMEM. With a gentle stream from a 1-mL pipet, straighten out the curled edges of the graft. Be careful to maintain the original orientation of the graft (see Note 3b).

3. After dispase treatment, grafting should be performed within 30 min. While preparing the animals for grafting, keep the graft moist in DMEM (1–2 mL/35-mm dish) at 37°C (10% CO₂).

3.3.2. Grafting Modified Epithelia

Several methods have been described for the transplantation of cultured human keratinocytes onto athymic mice. We prefer a model described by Barrandon et al. in which the cultured epithelium is grafted onto the inner side of a full thickness skin flap on the back of the mouse (22). By placing the epithelial sheet under the skin, the graft is protected from desiccation and wound contraction and the boundaries of the genetically modified graft are easy to identify. The grafting of genetically modified epithelia described in this section is a modified form of the model described by Barrandon et al. The entire surgical procedure must be performed on anesthetized animals using sterile conditions in a laminar flow hood. The grafting procedure requires about 30–40 min/mouse.

1. Anesthetize mice by intraperitoneal injection of 2,2,2-tribromoethanol (23 G 3/4-in. needle, 30 μL working solution per gram of mouse).

2. Disinfect the entire back of the mouse with ethanol (70%).
3. To prepare a skin flap on the back of the mouse, make a 2 x 2 cm L-shaped, full thickness incision (through the panniculus carnosus) starting at the lower back of the mouse. Grasp the free corner of the skin flap with forceps and retract the flap while dissecting it carefully from the thoracic wall. Loosen the internal connective tissue membranes and be careful not to damage the well-vascularized inner part of the flap.

4. Close the flap and remove the dispase-treated graft from the incubator. Carefully aspirate the medium off the graft, keeping the epithelium flat. Place a sheet of silastic over the graft so that the upper surface of the epithelium adheres to it. With fine forceps, grasp one corner of the silastic together with an edge of the epithelium and invert. The epithelium is now sitting on the silastic membrane with its basal side facing upward. If necessary, straighten out the edges of the epithelium with the blunt end of the forceps.

5. Peel back the skin flap and slide the silastic with epithelium onto the thoracic wall. The basal surface of the epithelium is facing upward. The preparation of the graft site and the insertion of the silastic is illustrated in Fig. 1.

6. Grasp the free corner of the skin flap with forceps and, in one continuous motion, gently place the skin flap over the epithelium. After the skin flap is placed over the epithelium, avoid any further manipulation of the flap to ensure proper contact of the epithelium to the inner side of the mouse skin (see Note 3c).

7. Close the incision with surgical clips. With forceps, pinch the edges of the incision to form lips and apply clips over the incision. When positioning the wound edges, minimize manipulation of the skin flap.

8. Lift the animal by grasping the neck and tail (maintain horizontal position) and place gently in the recovery cage. House animals one to a cage.

9. As early as 7 d, grafts form a well-stratified epithelium. After 10 d, grafts become macroscopically visible as a slightly raised area under the mouse skin. Clips detach spontaneously after 3–4 d.
10. To harvest the graft, sacrifice the animal by CO₂ asphyxiation. Make a U-shaped incision, starting at the lower back of the animal. With forceps, grasp the caudal edge of the skin flap plus the silastic, and carefully raise the flap by dissection.

11. As a result of a foreign body reaction, a thin fibrous membrane has formed over the silastic sheeting. Before taking off the silastic sheeting that covers the epithelium, completely remove this fibrous membrane by dissection. After the membrane is removed, grasp one edge of the silastic and gently peel it off the human epithelium, which will remain on the mouse skin. After removal of the silastic sheet, the engrafted human epithelium appears as a circular, well-defined, opaque area at the inner surface of the skin flap. Trace the area of the graft as a measure of the take of the genetically modified graft (Fig. 2).

12. The human epithelium and subjacent mouse tissue can now be prepared either for histology analysis or the epithelium can be processed for tissue extraction. For histological processing, place the biopsy in toto (human epithelium adherent to underlying mouse tissue) into fixative. Pin the mouse tissue (human epithelium facing upward) onto a thin wax plate to prevent curling of the edges. Figure 3 shows the histology of a section through a graft harvested 7 d after transplantation. At low magnification, the various layers of the sandwich graft are recognizable. Starting from the top layer, the sandwich is composed of mouse epithelium, mouse dermis, mouse subcutaneous fat, panniculus carnosus, mouse hypodermic, and human epithelium.

13. To detect proteins expressed in the genetically modified epithelium, a tissue extract of the grafted epithelium can be prepared. Harvest the epithelium as described in step 8 and remove the silastic sheet. Grasp one edge of the human epithelium with fine forceps and dissect the human epithelium from the underlying mouse tissue using sharp scissors. Snap-freeze the epithelium in liquid nitrogen and keep at −80°C until used. In a 4°C cold room, grind the frozen tissue
Fig. 3. (A) Human and mouse tissue in sandwich configuration 7 d after grafting at low magnification (H&E, ×100). (B) Human epithelium at high magnification. Stratification of the epithelium is well defined. Since the human epithelium is covered by the silastic membrane, the cornified layer does not exfoliate and is therefore thicker than normal (H&E, ×200).

to powder in a chilled mortar (−80°C overnight). Transfer the powder to an Eppendorf tube and resuspend in 300–400 μL of Tris buffer (25 mM). Sonicate the solution for 10 s at 50 W. Pellet the cell debris by centrifugation for 5 min at 15,000g and transfer the supernatant into a new vial. Levels of the expressed protein can be detected in tissue extract by ELISA or other protein analysis.

4. Notes

1. Cells must be actively dividing in order to be genetically modified with recombinant retroviruses. It is imperative that optimal cell proliferation be maintained throughout the entire experiment. Moreover, keratinocytes lose their growth potential after they reach confluence. Therefore, we recommend that the primary cultures of keratinocytes be passed onto the virus-producing feeder layer prior to reaching confluence.

   The effectiveness of genetic modification is also a function of the amount (titer) of recombinant retrovirus produced by the packaging cell line. The titers of different packaging cell lines, as well as different retroviral vectors, can vary significantly. It is important to use virus-producing cell lines with titers >10⁵/mL.

2. Poor clonal growth of primary keratinocytes and the overgrowth of primary cultures by human dermal fibroblasts might indicate problems with the feeder layer. To ensure optimal growth conditions for keratinocytes, 3T3-J2 cells or virus-producing cells should not be prepared more than 24 h prior to inoculation with keratinocytes. During the experiment, do not allow the feeder layer to deterio-
rate. Additional mitomycin C-treated feeder cells can be added if necessary. Fresh stocks of 3T3-J2 cells should be prepared on a regular basis (every 3–4 mo).

3. Common problems encountered with the grafting technique are opening of the incision after surgery, absence of the graft, and impaired graft take.

a. Premature opening of the wound within the first 24 h after surgery is caused by inaccurate closure of the incision with ligature clips. Be sure that the edges of the wound are brought together in parallel and the ligature clips are applied with enough force. Open wounds should be closed with sutures on anesthetized animals.

b. Absence of the graft after transplantation is most likely caused by grafting the epithelium in the inverted orientation (basal side of the human epithelium not facing the inner side of the mouse skin flap). Cultured epithelium has a polarity with basal proliferative cells on the side that was attached to the dish before dispase treatment. It is imperative that this side of the epithelium be applied to the inner side of the mouse skin flap. The graft will not take if the epithelium is inverted. Therefore, when washing the detached epithelium, be mindful at all times of the proper orientation of the graft.

c. Impaired graft take (adherence) of the transplanted epithelium is evident as poor differentiation of the epithelium or decreased graft size. Graft take is the function of many factors. It is important that the cells used to prepare the graft are healthy. Poor cultures (i.e., low plating efficiencies) will produce poor grafts. If the cultures are confluent for too long and form blisters, the grafts will be poor. The best grafts have been prepared from primary, secondary, or tertiary cultures. Problems during surgery can also contribute to poor grafts. Vigorous manipulation of the detached epithelium during the grafting procedure can traumatize this extremely thin and fragile structure. Although the detached epithelium contracts to approximately one-third of its original size, do not try to stretch the tissue and minimize manipulation of the epithelium during the grafting procedure. Dislocation of the graft by subgraft fluid collection or increased motion of the animal after grafting are additional reasons for impaired graft take. Allow some of the excess medium covering the graft to evaporate (30–40 s) before covering the epithelium with the mouse skin flap. This helps promote adherence of the graft to the flap.

References


Methods for Particle-Mediated Gene Transfer into Skin

Ning-Sun Yang, Dennis E. McCabe, and William F. Swain

1. Introduction

During the past 5 yr, particle-mediated delivery techniques have been developed as a physical means for gene transfer into various eukaryotic systems, including plants, insects, fish, and mammals (1–7). For mammalian somatic tissues, this technology, popularly known as the gene gun method, has been shown effective in transfection of skin, liver, pancreas, muscle, spleen, and other organs in vivo (3,4); brain, mammary, and leukocyte primary cultures or explants ex vivo (2,5–7); and a wide range of different mammalian cell lines in vitro (3,6,7).

Among the applications of gene gun technology, transfection of skin tissues of live animals has resulted in the most interesting findings, enabling a series of transgenic studies with both basic research and gene therapy applications. Briefly, high levels of transgene expression have been demonstrated by in vivo particle-mediated gene transfer of skin epidermal tissues. This result was shown to be highly reproducible for virtually all animal species tested, including mouse, rat, hamster, rabbit, pig, dog, and rhesus monkey (2–4,8,9,11). The adaptability of skin gene transfer across a whole range of experimental animal systems illustrates the power and versatility of the gene gun transfection method. Efficient delivery and expression of transgenes in skin tissues has been extended to a variety of reporter genes (e.g., luc, CAT, β-gal, and so on) (2,4) and candidate therapeutic genes (TH, hGH, hAAT (3,8,9), a number of cytokines (10), and viral antigens (11)), demonstrating the wide ranging applicability of this gene transfer strategy.

In vivo gene transfer to skin tissues has been used to develop genetic immunization technology (9,11), for gene therapy of subcutaneous tumors (10) and wound healing (8), for delivery of RNA as transgenes (12), and for in vivo
analysis of transcriptional promoters and other regulatory sequences in recombinant gene constructs (4,13).

Histological studies show that genes can be introduced into and expressed in different cell layers of epidermal or dermal tissues by adjusting the ballistic variables. These include the motive force for particle acceleration, gold particle size, pretreatment and manipulation of epidermal and dermal tissues prior to blasting, and DNA formulations for coating gold particles. In general, topical skin gene delivery results in high-level transient expression almost exclusively in the superficial epidermal cell layers (3,4). Treatment of dermal tissues via exposure of the underside of a skin flap can result in long-term transgene expression, though at a much reduced level (4). Since the epidermis is exquisitely competent for eliciting both humoral and cell-mediated immune responses (9,11) and highly efficient in synthesis and secretion of transgenic proteins as well as easily accessible, it appears to be an attractive target for gene transfer; particularly for vaccine and cancer immunotherapy approaches. Mechanisms involving different cell types in transgene expression, secretion, and genetic immunization may now be effectively studied using the gene gun approach for transgenic studies of skin.

Several gene gun designs were developed for particle-mediated gene transfer in eukaryotic systems (1,2). For in vivo DNA delivery into mammalian somatic tissues, virtually all the reported studies have employed one of two hand-held device models: an electric discharge/Accell design or a helium-discharge/Biolistics design. More recently, a new hand-held device has been developed (the Accell pulse gun) that uses a helium jet for particle acceleration. These three types of gene gun have all been shown to efficiently deliver DNA to the epidermis.

In this chapter, we will describe the general principles, mechanisms, protocols, and uses of the particle-mediated gene transfer technology for gene transfer into skin tissues in vivo. Specific applications of this technology to basic molecular biology as well as gene therapy and genetic immunization will be discussed.

1.1. Hardware Designs

Particle-mediated gene transfer is a physical means for gene transfer and was originally developed for gene transfer to plant cells (1,14–16). It is also known as the microprojectile, biolistic, or gene gun method. With particle-mediated gene transfer, microscopic gold particles are coated with transgenes of interest and accelerated by a motive force to sufficient velocities to penetrate the target cells, resulting in intracellular delivery of the DNA molecules. The motive force can be generated by a high-voltage electric discharge (Accell), a helium pressure wave (Biolistics), or by other means (17). The
Accell high voltage-discharge device requires a large capacitor and switching capable of quickly transferring a 10 μF, 5–25 kV charge to arc points. The Accell device is a relatively large instrument (3 x 2 x 2 ft), mainly housing the necessary electrical components. A highly valuable feature of the Accell electric device is that the shockwave for particle acceleration can be fine-tuned by adjusting the discharge voltage (2–4). This device was designed and has been continuously modified by D. McCabe (Agracetus, Middleton, WI) and has been used effectively by a number of researchers working on particle-mediated gene transfer (1–8,10–13).

The Bio-Rad (Hercules, CA) Biolistics device employs a small cylinder and a rupture membrane (~2 in. in diameter and 10 in. in length) to generate and regulate a shockwave from compressed helium (9,19). More recently, a new helium pulse gun has been developed (Accell User’s Manual, Auragen), and its improved features for particle-mediated gene transfer to skin will be described (Fig. 3A; Notes 3–5). For transgenic studies involving most experimental animals, all three types of hand-held gene guns apparently can perform similarly well for skin tissues in vivo. Specific advantages may exist for one type over another, depending on the special need for certain experimental conditions.

1.2. Mechanics of the Particle-Mediated Gene Transfer Devices

The particle acceleration mechanism of the original Accell gene gun device is diagrammed in Fig. 1. In principle, microscopic gold particles are coated with DNA in a precipitated, dry form in an ethanol suspension, and evenly deposited onto a carrier sheet. A strong shockwave, generated by the high voltage electric discharge, then accelerates the mylar sheet carrying the DNA/gold particles to a very high velocity, resulting in controlled penetration of target tissues (e.g., skin) placed in the pathway of particles, effecting efficient intracellular delivery of DNA. Other technical details for gene gun operation are described in the legend of Fig. 1. Whereas the energy source for generating a shockwave using the Biolistics helium pressure gun (PDS 2000) is different from Accell’s energy source, the mode and mechanism of accelerating a kapton membrane sheet carrying predeposited, DNA-coated gold particles for transfecting target cells (9,19) is somewhat similar to that described in Fig. 1.

1.2.1. Hand-Held Gene Gun Device

An improved design of the hand-held Accell gene gun device is shown in Fig. 2. A cassette mode gene tab (containing DNA/gold particles on the mylar sheet) and a retainer tab are designed to facilitate efficiency in gene transfer experiments.

1.2.2. Mechanism and Design of an Accell Helium Pulse Gun

More recently, D. McCabe (at Auragen) has developed a helium pulse gun that establishes a new mechanism of particle acceleration, and is diagrammed in
Fig. 1. The motive force is generated in a spark discharge chamber containing two electrodes. A 10-μL water droplet is placed in between the electrodes, and a high-voltage capacitor is discharged through the water droplet that vaporizes instantly, creating a shock wave. We have found that a polyvinyl chloride pipe with an internal diameter of 13 mm is adequate for use at the spark discharge chamber. The electrodes are located opposite each other, project into the interior of the chamber approx 5 mm below the top, and are protected at the tips with an arc-resistant alloy. The gap between the two electrodes can be adjusted by appropriately threading them into or out of the spark chamber. A spacer ring is placed above the spark chamber that, in a fixed apparatus for transformations of a single crop species, can be a vertical extension of the spark discharge chamber. However, a removable spacer ring allows the distance from the spark discharge to the carrier sheet to be varied so that the force of the shock wave can be adjusted. The motive force can also be adjusted by varying the voltage of the discharge. The carrier sheet on which the DNA-coated gold particles are precipitated is placed on top of the spacer; the function of this sheet is to transfer the force of the shock wave from the spark discharge into acceleration of the carrier particles. Located above the carrier sheet is a 100-mesh stainless steel screen that retains the sheet so that it does not proceed to the target tissue. The target tissue can be placed on Petri plate in such a way that when the plate is inverted over the retaining screen, the tissue is in the direct path of the gold particles. The whole assembly is under a partial vacuum in order to minimize aerodynamic drag. (Reprinted from ref. 16.)
1.2.3. Operational Parameters for Gene Transfer

Functional parameters that can be modified to optimize particle-mediated gene transfer to skin include the following: physical acceleration rate for particle penetration; shape, form, and size of particles; particle loading rate per target area; and DNA loading rate per particle. For transfecting appropriately treated skin tissues, a discharge voltage of 15–22 kV for the Accell device (4) and a pressure of 300–500 psi for the helium pulse gun (McCabe, D., Burkholder, J., and Yang, N.-S., unpublished results) have been found to confer high levels of transgene expression in mouse skin. Particles made from dense materials, including gold, tungsten, iridium, and platinum are all capable of effective coating and delivery of DNA via gene gun gene transfer. However, gold particles are most commonly chosen for two reasons: elemental gold is chemically inert with no cytotoxic effects, and owing to its use in the electronics industry, uniform sized μm-scale gold particles are commercially available.
Fig. 3. Design, operation, and function of the Accell helium-discharge pulse gun. (A) Diagram of the Accell helium pulse particle-mediated gene delivery mechanism (Courtesy of D. McCabe); A, helium reservoir; B, release valve; C, DNA/gold cartridge; D, exit nozzle; E, accelerating gold particles coated with DNA; F, target tissue. (B) Accell helium pulse gun gene transfer to mouse skin tissue. Epidermal transfections can be conveniently performed at a rate of 5 s per mouse. (C) Particle-mediated gene transfer to canine oral mucosal tissue. (D) Transgene expression of pCMV Lac-Z DNA delivered using the helium pulse gene gun into surgically exposed subcutaneous mouse tumor tissues. Histological data shown here demonstrates that the 1–3-μm DNA-coated microscopic gold particles can effectively penetrate 20–30 cell layers and deliver transgenic DNA into targeted tumor cells. The transfection was performed into the dermal side of the skin flap, which was reflected surgically before gene delivery.

A wide range of gold particle sizes have been evaluated for gene transfer via particle-mediated gene transfer. These include 0.95-, 1–3-, 5–7-, and ≥15-μm diameter gold particles. For mammalian skin tissues, the 0.95- and 1–3-μm gold particles are recommended (3,4,11). Some gold particles are commercially available in different forms (e.g., as round particles, crystals, or even aggregates). For gene transfer to skin epidermal cells, crystal and spherical gold particles were found to be similarly useful in gene delivery.
A major advantage of the particle-mediated gene delivery method for gene transfer is its capacity for in vivo intracellular delivery of high copy numbers of DNA into target tissues. Onto a single 1–3-µm gold particle, more than 10,000 copies of 5–10 kb plasmid DNA (but usually only 1000–5000) can be effectively coated with Ca\textsuperscript{2+}/spermidine or PEG formulation precipitated from form (7,14,19,20). With a predetermined gold particle loading rate of 0.1 mg/cm\textsuperscript{2} approx 1–2 gold particles (1–3 µm) per cell can be delivered via random distribution into the epidermis containing stratified epithelial cells on the order of ~15 µm in diameter. Excessive particle loading rates (e.g., ≥5 particles/cell) can often cause trauma to transfected tissues. On the other hand, too low a particle load (e.g., 1 particle/3 cells) often results in low gene transfer efficiency. A preliminary gene transfer to any tissues using reporter genes can effectively determine the optimal particle loading rate and DNA dosage for specific skin gene transfer systems. Use of a reporter gene capable of histological assay greatly facilitates optimization of transfection efficiency.

Another important feature of particle-mediated gene delivery is the absence of restriction on the size of the DNA vectors. We and others (2,18) have shown that plasmid DNA, genomic DNA (≥23 kb), and reporter genes cloned in lambda phage genomic libraries (~44 kb) can all be effectively delivered by gene gun techniques for transgene expression in transfected mammalian cells. This capability offers new opportunities for transferring multiple genes, large size genomic DNA gene(s), or multiple tandem genes into mammalian somatic tissues. In addition, cotransfection of multiple genes on different plasmids has also been shown to be efficiently achieved by using the particle-mediated gene transfer method (21). Furthermore, we have recently shown that RNA molecules can be similarly delivered as DNA vectors via gene gun technology (12).

2. Materials
2.1. Instrumentation

The Accell electric-discharge gene gun is available through collaborations with Agracetus (Middleton, WI). The PDS 2000 Biolistics helium pressure device is commercially available from Bio-Rad (9,19,22). The newly developed Accell helium pulse gun will likely become commercially available (from Bio-Rad) in the future. Mechanical operation and/or manipulation of the gene gun devices are relatively straightforward, as detailed by the manufacturer’s manuals.

2.2. Elemental Gold Particle Preparations

Microscopic gold particles of different sizes (e.g., 0.95, 1–3, 5–7 µm, and so on) and in different shapes (e.g., spherical particles, crystals, aggregates, and
so on) can be purchased from Degussa Corporation (South Plainfield, NJ). It is important that these gold particles be obtained as elemental gold, not as gold salt or colloidal gold. If necessary, the gold particles can be washed and cleaned by rinsing them in distilled water, 70% ethanol, and 100% ethanol in sequence prior to use. The particles can also be sterilized in phenol or CHCl₃ if necessary. It is important to microscopically examine each newly purchased gold particle preparation, making sure that the lot, particle size, and form are correct and appropriate as desired for the test systems. It is also useful to test each new lot of gold particles in a gene transfer and expression assay as some lots do not perform satisfactorily for reasons that are currently unknown.

2.3. DNA Vectors

A clean plasmid DNA preparation purified from CsCl gradient or Qiagen (Chatsworth, CA) columns, ethanol precipitated, and redissolved in TE buffer (10 mM Tris-HCl, pH 7.0, 1 mM EDTA), dilute EDTA (0.1 mM), or distilled water should be used for coating particles. We have not observed significant differences in transfection efficiencies between supercoiled plasmids and linear DNA in contrast to published reports using other methods. Genomic DNA preparations, genomic DNA clones in lambda phage or cosmid libraries, or cocktails of different DNA vector systems or preparations can be mixed in desired molar ratios in aqueous solution, and then loaded onto gold particles. For exploratory gene transfer experiments, convenient reporter gene systems that have low endogenous background, have a sensitive assay, and are inexpensive are recommended for verification of transient gene expression systems. A vital assay is a distinct plus.

2.4. Reagents for Coating DNA onto Gold Particles and Gene Transfer

1. 0.1M spermidine in H₂O. Use only fresh spermidine made weekly from a free base solution (Sigma).
2. 2.5M CaCl₂ in H₂O.
3. 50% PEG (mol wt 3000-4000) in H₂O.
4. 100% EtOH at -20°C.

2.5. Materials for Loading Carrier Sheet

1. 0.5-mL mylar sheets (1.8 cm²).
2. Coated particle preparation suspended in EtOH.
3. Desiccant.
4. Vacuum desiccator.
5. Vacuum pump.

2.6. Reagents for Treatment and Care of Skin

1. Electric hair clippers.
2. Depilatory (i.e., Nair).
3. RPMI-1640 with 1 μg/mL gentamicin solution.
4. Tegaderm adhesive (3M).
5. PBS.
6. Anesthesia: ketamine 70 mg/kg and xylazine 17 mg/kg.

2.7. Tissue Extraction Buffers and Enzyme Assay Systems

2.7.1. Luciferase Assay

1. Extraction buffer: 34 mL H₂O, 10 mL 0.5M potassium phosphate buffer (pH 7.5), 5 mg BSA, 6 mg Pefabloc SC, 5 mL 10% TX-100.
2. 5X reaction buffer: 0.46 gm glycylglycine, 0.7 gm MgCl₂·6H₂O, add to 20 mL H₂O while stirring; 0.540 gm ATP; 0.225 mL 2M Tris-HCl pH 7.5. Adjust pH to 7.8, bring up to 30 mL, then add 15 mg BSA.
3. Luciferin solution: dissolve 14 mg luciferin in one drop of 1M NaOH. Immediately add to 50 mL H₂O with 566 μL 7.5% NaHCO₃. Adjust pH to 6.0. Protect from light.

2.7.2. CAT Assay

1. CAT buffer: 4.275 gm sucrose, 3.125 mL 2M Tris-HCl (pH 7.5), 0.25 gm ascorbate (Na salt), 0.25 gm cysteine (free base), 6.2 mg leupeptin, 0.5 mL 0.5M EDTA. Bring up to 25 mL with distilled H₂O and pH to 7.5.
2. Hot mix: 50 μL ¹⁴C-chloramphenicol, 200 μL 50 μM acetyl CoA, 150 μL distilled H₂O.
3. Ethyl acetate.

2.7.3. X-Gal Assay

1. X-gal substrate (5-bromo-4-chloro-3-indolyl-β-d-galactoside): Dissolve X-gal into dimethyl formamide at a concentration of 40 mg/mL.
2. Buffer solution: 44 mM HEPES buffer, 3 mM K⁺ ferricyanide, 3 mM K⁺ ferrocyanide, 15 mM NaCl, 1.3 mM MgCl₂, pH 7.4.
3. X-gal buffer: Add X-gal substrate to buffer solution to make a final 1 mg/mL solution.

2.7.4. Cytokine Assay and Other Assays

General extraction buffer: 9.5 mL PBS (0.2 gm potassium chloride, 0.2 gm potassium phosphate monobasic, 8.0 gm NaCl, 1.15 gm sodium phosphate dibasic to 1 L distilled H₂O), 2.4 mg Pefabloc, 0.5% Triton X-100.

3. Methods

3.1. Coating DNA onto Gold Particles

1. Purified plasmid DNA or other DNA vectors via CsCl gradient centrifugation or Qiagen columns followed by ethanol precipitation and redissolved in TE buffer.
2. Prepare DNA solution at a concentration of 1 µg/µL, and store at 4°C.
3. Precipitate the DNA onto the gold particles: Weigh approx 10 mg of gold particles into an Eppendorf tube. Add DNA at the desired DNA/gold ratio and bring volume up to 100 µL with distilled H₂O. Add 100 µL 0.1M Spermidine and mix gently. Add 100 µL of 2.5M CaCl₂ quickly while vortexing. Continue vortexing for 15 s. Incubate at room temperature for 10 min, then centrifuge briefly. Remove supernatant and wash particles with 100% EtOH by gently vortexing and again centrifuge briefly. Resuspend particles at the desired suspension ratio in 100% EtOH. Sonicate briefly to disperse particles. Particle suspensions can be used immediately or stored at 4 or -25°C if precautions are taken to exclude moisture. As an alternative, the DNA can be precipitated using 50% PEG and 1.0M CaCl₂ (7).
4. DNA dosage: For transfecting mammalian skin tissues, DNA dosages at 0.1, 0.5, 1, and 2.5 µg plasmid DNA/mg particle have been found to exhibit a linear relationship with transgene expression levels.

3.2. Deposit DNA/Gold Particles onto Carrier Sheet
1. Carefully cut clean mylar sheets into a uniform size (1.8 cm²) for the Accell device using a sharp razor blade.
2. Place a single mylar sheet into a 60-mm dish.
3. Vigorously shake DNA particle suspension and pipet 324 µL onto the mylar sheet, being careful not to break the meniscus.
4. Shake the particle preparation again and repeat dispensing onto a different sheet.
5. Load less than 8 sheets at a set.
6. Allow the particles to settle out for 1–3 min and adhere to the sheet.
7. Break the meniscus and place the sheet onto a larger plastic dish with the rest of the set and immediately place them in a desiccator and pull a vacuum until the sheets are completely dry (~2 min).
8. The DNA-coated sheets are ready to use or can be stored at 4°C under desiccation.

3.3. Preparation of the Particle-Mediated Gene Transfer Device
1. Place mylar sheets loaded with DNA-coated particles onto the propulsion platform.
2. Turn on the capacitor and dial to the desired discharge voltage.
3. Appropriately place the targeted skin over the particle pathway.
4. Simultaneously press the safety release and discharge trigger to transfect the skin.
5. Other information on manipulating the Accell electric-discharge device is described in Fig. 1.

3.3. Animal Care and Skin Treatment
1. Small experimental animals (including mouse, rat, hamster, rabbit, and so on) are anesthetized using a mixture of ketamine and xylazine as appropriate for species. Large animals including dogs, pigs, and rhesus monkeys are anesthetized under the guidance of the consulting veterinarian as required by the Animal Welfare Act and administered by the institution's IACUC.
2. Animal hair in the target area is removed with clippers. A depilatory such as Nair can be used to remove residual stubble, but this treatment is not necessary. If using a depilatory, care should be taken not to allow prolonged treatment, as this may damage the target tissue.

3.4. Epidermis and Dermis Gene Transfer

1. Exposed epidermal cell layers can be effectively transfected at a gold particle loading rate of 0.2-, 0.1-, 0.05-, or 0.01-mg particles (1–3-μm or 0.95-μm diameter) per cm² of skin tissue, the exact rate being determined experimentally. In general, a 0.1-mg particle/cm² is highly effective for most animal species.

2. With the Accell device, apply a set of discharge voltages (15, 18, 22, and 24 kV) for optimization of epidermis gene transfer. Eighteen kilovolts is often found to be optimal for rodent epidermal systems, but a matrix of voltage and particle rates should be run since these interact.

3. To transfect dermal tissues, make an incision and dissociate the full thickness skin tissue of the target size (~3 × 2 cm²) from the facia and muscle tissue using standard surgical procedures and tools. Flip the skin flap over, exposing the dermal tissue and transfect the fibroblasts, muscle cells, and other stromal cell types at 15–24 kV. Moisten dermal tissues with sterile saline before closing. Close incision with sutures or wound clips.

4. If desired, apply semioclusive skin dressings such as Tegaderm (3M, St. Paul, MN) or Opsite (Smith and Nephew, Hull, UK) to transfected epidermis. This application often results in higher transient gene expression.

5. Sacrifice the animals at designed time points after gene transfer. Excise the skin target and assay for transgene activities or sequences as described in Section 3.5

3.5. Reporter Gene Expression Assays

3.5.1. Tissue Extraction

1. Collect transfected skin tissues from test animals by excising a small uniform portion of the 3.24 cm² targeted surface area. A piece as small as (1–2 mm²) can be enough for Luc, β-gal or CAT reporter gene expression assays.

2. Drop the freshly excised skin piece into the appropriate buffer (1:1, wt:vol) and freeze in liquid nitrogen.

3. Immediately before performing the assay, grind, scissor mince, or homogenize the frozen skin, keeping it as cold as possible. Avoid foaming the tissue.

4. Centrifuge the sample at high speed to separate the tissue from the lysate. Soluble crude tissue extracts are used in microliter quantities for various reporter gene assays.

3.5.2. Luciferase Assay

1. After lysate has been collected, prepare dilutions in PBS if necessary.

2. Add 80 μL reaction buffer, 1–10 μL lysate, and 310 μL distilled H₂O into a reaction tube and vortex.
3. Read relative light units in a luminometer (e.g., Lumat LB 9501, Wallac, Gaithersburg, MD) and run a standard curve to quantify results

3.5.3. X-Gal Staining

1. Perform a whole mount tissue staining by simply placing the excised skin target into X-gal buffer. For best results, we suggest gluing the skin to a 35-mm dish to keep the skin stretched out for better examination. The tissue can also be fixed in methanol:acetone (1:1) for 10 min and then stained with X-gal buffer as described by MacGregor et al. (23).

2. For microscopic examination at the cellular level, make tissue sections using a cryostat microtome or paraffin sectioning. After the tissue has been sectioned into ~10-µm-sections, place the slide in ice-cold 1.5% glutaraldehyde solution for 10 min, and wash in ice-cold PBS 5 times for 5 min each. The tissue slides are then stained with X-gal buffer for 1 h. Avoid prolonged staining because a blue background can develop in hair follicles of certain skin tissues.

3.5.4. ELISAs and RIA

Enzyme-linked immunosorbent assay (ELISA) tests are most commonly used for cytokine quantification. They may be purchased as a kit or antibody pairs may be purchased, and used in a sandwich-style assay (i.e., capture Ab-cytokine-detecting Ab-conjugate-substrate). The ELISAs may be run on serum, plasma, skin extract (using general extraction buffer), or tegaderm-stripped cell extract. Some transgenes such as hGH can be detected using a radioimmunoassay (RIA). This assay can also be used with serum, plasma, skin extract, or tegaderm extract.

4. Notes

1. Particle-mediated gene transfer provides a physical means of delivering biologically active molecules, including DNA, RNA, and proteins intracellularly. Because of its physical nature, this method displays properties distinct from those characteristic of chemical and biological gene transfer agents, and it may prove advantageous in many cases. The method is readily adaptable to a wide range of tissue and cell types, from walled fungal cells and plant cells in organized meristematic tissues (1,14) to various mammalian cells and organs (2,18). A major advantage of particle-mediated gene transfer is its applicability to cells in vivo. Its effectiveness has also been demonstrated in several animal species including natural populations that are genetically heterogeneous, which is especially significant in extrapolation of experimental results in laboratory animals to human populations.

2. No restrictions on the size or form of the DNA transferred have been encountered thus far, other than those inherent in the need to prepare the DNA and perform routine manipulations, like pipeting. The amount of DNA required for particle-mediated gene transfer is relatively low; for example, nanogram amounts of DNA per cm² of target area have been shown effective for vaccine applica-
Particle-Mediated Transfer to Skin

This amount corresponds to 100–1000 plasmid molecules per particle, on average, assuming a uniform distribution of DNA on the particles. In other studies, an amount of 5000 copies of cDNA delivered per cell was found to be optimal for commonly used reporter genes (3, 4, 18).

3. The current disadvantages of particle-mediated gene transfer in vivo are the limited transfection efficiency for certain tissue systems (particularly if permanent gene transfer to the target cells is necessary) and the depth of tissue that can be accessed. Although transient transfection efficiencies from 5–15% can frequently be obtained in vivo, and efficiencies approaching 30–50% are possible in vitro, efficiencies for stable (i.e., integrative) gene transfer in vivo are apparently low, and have not been clearly established in various transfected somatic tissues. Long-term transgene expression following particle-mediated gene transfer has been observed in muscle and dermis, but these tissues seem to be the exception rather than the rule. More general long-term gene expression may be possible through a combination of particle-mediated gene transfer and replicating or actively integrating vector systems, but for the present it appears that the technique is most suitable in applications where short- to medium-term transgene expression is sufficient or desirable.

4. The current devices for particle delivery can achieve penetration depths up to a few hundred micrometers depending on the target tissue, corresponding to a depth of 20–30 cell layers in a stratified tissue like the epidermis. This depth of penetration, though not sufficient to reach internal organs, is greater than that typically achieved with viral vectors or chemical gene transfer agents where penetration is often limited to only one or two cell layers. The gene gun method at present also cannot deliver gene systematically to cell fractions scattered in large, three-dimensional tissues like liver or brain as can certain other gene transfer agents that can be administered through the circulatory system (26).

Skin is an attractive target tissue for gene therapy applications, and appears particularly amenable to particle-mediated gene transfer. One striking advantage of skin is its accessibility. One can conduct gene transfer to skin without resorting to invasive procedures. The organization and dimensions of the epidermis are also a good fit with the penetration capabilities of the current particle acceleration devices.

A second advantage of skin derives from its continuous turnover. A vast majority of epidermal keratinocytes are differentiated nondividing cells destined to eventually exfoliate from the body. Gene transfer to these cells is thus temporary and self-reversing, unless the gene transfer itself disrupts their normal differentiative process. Gene transfer to the basal cells that repopulate the epidermis, although conceptually possible, is currently unlikely owing to the scarcity of these cells and their position in the deepest regions of the epidermis. We have not yet observed sectors of permanent transgenic epidermal cells as a result of epidermal gene transfer. Approaches seeking to transfect these generative cells to produce stable transgenic skin will thus probably require special targeting strategies to be successful.
5. Particle-mediated gene transfer to skin tissues of various animal species results in high level expression of reporter genes (4). Epidermal gene delivery using cytokine genes resulted in reduction or prevention of growth of subcutaneously transplanted tumors in mouse models (10). In this study, high levels of cytokines were detected in transfected tumor and neighboring normal skin tissues, and much lower but significant levels were also detected in serum of test animals. In addition, gene transfer to keratinocytes has been employed to produce therapeutic proteins locally, e.g., to produce growth factors to promote healing in wounds (8). Systemic distribution of endogenous proteins produced in skin has been reported (25). Future augmentation of transgene expression may extend this observation more effectively to transgenic systems.

6. The epidermis plays a key role in providing first-line immunological responses. The immune and inflammatory processes now clearly demonstrated in cutaneous tissue make it a suitable target for gene transfer-based vaccines. In addition to keratinocytes, the epidermis contains Langerhans cells and dendritic epidermal T-lymphocytes, both of which serve as professional antigen presenting cells. Particle-mediated gene transfer to skin has been shown to result in induction of high level and durable humoral and cytotoxic immune responses (9, 11, 24). These immune responses were shown to be fully protective in immunized mice subjected to a lethal challenge with influenza virus (11). These studies demonstrate the usefulness of particle-mediated gene transfer to skin in the context of a prophylactic vaccine, and suggest the potential of a similar immuno-therapeutic approach to infectious disease and cancer.

7. The use of mRNA, ribozymes, or oligoribonucleotides to transfer genetic or biochemical information into somatic cells in vivo or in vitro may be advantageous in certain gene therapy protocols because RNA molecules do not integrate into nuclear DNA under normal cellular physiology, hence eliminating the possibility of insertional mutagenesis in transgenic cells. Qiu et al. (12) showed recently that the particle-mediated gene transfer technology offers good opportunities for these gene transfer/gene therapy approaches. The particle-mediated gene transfer method for gene delivery to skin tissue may also provide an efficient and convenient system for basic research in posttranscriptional, translational, and ribozyme regulations in mammalian somatic cells in vivo.

References


Methods for Liposome-Mediated Gene Transfer to Tumor Cells In Vivo

Gary J. Nabel, Zhi-yong Yang, and Elizabeth G. Nabel

1. Introduction

Despite considerable progress in the understanding of the molecular basis of cancer, many malignancies remain resistant to traditional forms of treatment. Certain cancers, such as melanoma or renal cell carcinoma, respond to modulation of immune function, presumably because the immune system is induced to recognize mutant proteins in tumor cells. The advent of cationic liposomes as safe and increasingly efficient vectors for gene transfer in vivo has allowed the development of new approaches to immunotherapy that offer alternative therapies for cancer in the future.

Cationic liposomes represent synthetic genetic delivery systems that avoid the potential infectious complications of viral vectors. Liposomal delivery of nucleic acids to the cytoplasm to cells allows them to overcome lysosomal degradation with subsequent delivery to the nucleus. The efficiency of DNA encapsulation into conventional anionic liposomes is low since the volume of conventional liposomes is small relative to plasmids. Transfection efficiencies are also poor for this reason, and because conventional liposomes do not efficiently bypass lysosomal degradation. In contrast, cationic liposomes, also called cytofectins, were developed to overcome these encapsulation and transfection deficiencies. Studies employing cationic liposomes encapsulating DNA have shown site-specific transfection in humans in vivo. In vivo gene transfer with DNA-cationic liposome complexes is proving to be safe to the host, lower in cost because of the relative ease of preparation, and applicable to the treatment of cancer. Cationic liposomes are now finding clinical application in several human gene therapy protocols for the treatment of malignant
melanoma, colon, and other cancers. Development of new liposomes offers potentially more effective gene delivery than originally described compounds.

Positively charged lipids form complexes with DNA by interacting with the negatively charged phosphodiester backbone. This electrostatic interaction is highly dependent on the molar ratio between the DNA and the liposome, and occurs spontaneously. Ideally, the complex incorporates the DNA completely, regardless of its size. DNA liposome complexes assemble spontaneously in aqueous solutions without extensive preparation, making it possible to analyze different vectors in vitro or in animal models.

The ratio of cationic and neutral lipids to DNA is the most important determinant of transfection efficiency. When the amount of positive charges contributed by the liposome equals the number of negative charges on the DNA, the DNA liposome complex will be neutral. Titration of DNA to liposome therefore determines its overall charge and thus its uptake by cells. Differences in the type of target cell, plasmid, and mixture making up the liposome complex require individual adjustments to the DNA-to-lipid ratio. With all methods of cell transfection, expression levels and transfection efficiencies depend on the plasmid expression vector, cell line, and host cell. Cationic liposomes are also often inactivated by polyvalent, negatively charged serum components, and serum-free conditions at the transfection site are important for the successful use of catheter-based delivery. Direct gene transfer of cationic liposomes with either catheter-based delivery or direct injection has shown gene expression limited to the site of gene introduction. Plasmid DNA in liposome complexes is unlikely to integrate or propagate in animal cells. Cationic liposomes have also been well-tolerated in vivo, and biochemical, hemodynamic, or cardiac toxicities have not been observed.

The prototype cationic lipid for gene transfer was DOTMA (N[1-{2,3-dioleoyloxy}propyl]-N,N,N-tri-methylammonium chloride), which was mixed with an equimolar amount of DOPE (dioleoyl phosphatidylethanolamine). The lipid DOTMA/DOPE comprised the cationic liposome commercially known as Lipofectin (Bethesda Research Laboratories, Gaithersburg, MD). Lipofectin was used in a number of laboratory studies, but has not been utilized in human gene transfer clinical protocols. For human studies, two different cationic liposome formulations have been used. The first includes DC-cholesterol (3b[N-(N'N'-dimethylaminoethane)-carbamoyl] cholesterol) mixed with DOPE. DC-cholesterol/DOPE in low concentrations has proven to reduce toxicity to cells in vitro, is metabolized in vivo, and has provided successful gene transfer into malignant tumors in humans. At high absolute concentrations of DNA, however, these liposome complexes have led to aggregation and in vivo toxicity following iv injection in mice, which limited the doses that could be delivered.
More recently, a cationic liposome comprised of dimyristoylphospho-
3-dimethyl-hydroxyethyl ammonium (DMRIE) with DOPE has allowed up to
100-fold higher concentrations of lipid and DNA to be administered in vivo
with minimal toxicity. In vitro studies of the comparative efficiency of trans-
fection with DC-Chol/DOPE and DMRIE/DOPE in different cell lines showed
improved transfection efficiencies of two- to seven-fold with DMRIE/DOPE,
with the greatest improvement in cell lines which are difficult to transfect, such
as the MCA 205 fibrosarcoma.

This improvement may be owing to the higher concentration of plasmid
DNA used with the cationic liposome. In previous studies, the higher concen-
tration of plasmid was toxic to cells when used with DC-Chol/DOPE. Analyses
of the biochemical and pathologic changes in pigs and mice after administra-
tion of DMRIE/DOPE with DNA concentrations up to 1000-fold proposed for
human trials showed no adverse effects. Thus, although DMRIE/DOPE may
not necessarily be more potent than DC-Chol/DOPE, DMRIE/DOPE showed a
lack of toxicity that allowed larger quantities of DNA to be used. Additional
improvements in lipid chemistry and, possibly, in cell targeting will facilitate
the development of specific and efficient gene transfer in vivo.

Because of its greater ease of preparation and administration, direct gene
transfer, rather than cell-mediated or ex vivo gene delivery, represents a prom-
ising technique for clinical applications. Cationic liposomes have several
desirable features for direct gene transfer. Because there are fewer constraints
on packaging, different enhancer/promoter elements or various sized plasmids
can be used. In contrast to retroviral vectors, division of the target cell is not
necessary, allowing recombinant gene expression in nondividing cells. Expres-
sion is site-specific, minimizing concerns about widespread dissemination of
the vector. Thus, DNA liposome complexes are applicable for delivery to focal
sites of disease. Target cells include vascular endothelial and smooth muscle
cells of the tumor microcirculation or tumor cells themselves.

A characteristic feature of the pathologic lesions of many human diseases is
the abnormal expression of genes focally. In cardiovascular disease, for
example, growth factors, cellular adhesion, procoagulant, and other gene prod-
ucts stimulate formation of atherosclerotic plaques that can ultimately have
profound effects on myocardial perfusion. The focal lesions of cancer are
tumors, against which the immune system can potentially provide protection.
The immune system can contribute to the surveillance and destruction of neo-
plastic cells, as shown in a number of studies in which cellular and humoral
immune effectors inhibit tumor cell growth. Studies have shown that the
expression of highly immunogenic class I major histocompatibility complex
(MHC) antigen is often lacking or decreased in freshly isolated cells from natu-
 rally occurring tumors. Deficient expression of class I MHC molecules may
limit the present action of tumor antigens to cytotoxic T-cells, thus facilitating tumor cell growth. Several tumor cell lines exhibiting low levels of class I MHC proteins have been shown to become less oncogenic when expression vectors encoding the relevant class I MHC antigen were introduced into them. Direct in vivo transfection of this antigen into tumors via DNA/liposome complexes may provide an effective immunotherapy against cancer. In particular, the introduction of foreign histocompatibility antigens may induce an allogenic response locally, which can enhance local immunity and lead to improved immune recognition of tumors. The additional delivery of cytokine or other genes, including growth inhibitors, antiangiogenic factors, or prodrug converting enzymes, may further enhance this process and provide additional approaches to utilize direct gene transfer to enhance antitumor responses.

2. Materials

2.1. Vector Production and Analysis

A eukaryotic expression vector plasmid, pHLA-B7, is prepared by insertion of an HLA-B7 gene cDNA into the Rous sarcoma virus (RSV) RSV β-globin plasmid (1,2). The β-globin gene is removed by digestion with HindIII and BglII, treated with calf intestinal alkaline phosphatase, phenol and chloroform extracted, ethanol precipitated, and treated with the Klenow fragment of Escherichia coli DNA polymerase to generate the plasmid backbone. An insert is prepared as a BamHI to SalI fragment of pLJ-HLA-B7 (3), kindly provided by Dr. Alan Korman, which is treated with Klenow enzyme and ligated to the fragment from RSV-β-globin. The resultant plasmid contains pBR322, the RSV enhancer, and the SV40 polyadenylation sequence, similar to RSV-β-globin (1). Plasmids are grown and purified in the absence of the ethidium bromide or penicilllin derivatives, using a commercially available column chromatography method (Promega, Madison, WI).

2.2. HLA-B7/β-2 Microglobulin Plasmid Expression Vector

The expression vector for the class I MHC gene is prepared as follows: the HLA-B7 gene is inserted into a simplified eukaryotic expression vector that utilizes the RSV enhancer to stimulate expression of the HLA-B7 gene. A second open reading frame is included using an internal ribosome initiation site derived from encephalomyocarditis virus (ECMV). This plasmid uses a bovine growth hormone poly A site, eliminating all viral processing and polyadenylkation sequences. The plasmid DNA is grown in a standard E. coli host strain (DH5α or XL1-blue). This vector has been constructed by insertion of the HLA-B7 gene cDNA into the RSV β-globin plasmid. Briefly, the β-globin gene has been removed from this plasmid by digestion with HindIII and BglII treated with Klenow fragment of DNA polymerase and used as the
backbone to insert the \textit{Bam}HI to \textit{Sal}I fragment of HLA-B7 treated with Klenow enzyme. This plasmid contains pBR322, a kanamycin resistance gene, the RSV enhancer, HLA-B7, ECMV ribosome entry site, $\beta$-2 microglobulin, and the bovine growth hormone polyadenylation sequence.

### 2.3. Cationic Liposomes

We have used two cationic liposomal preparations for our in vivo human gene therapy trial. In the first trial (4), dimethylaminoethane-carbamoyl cholesterol (DC-Chol)/dioleoyl phosphatidylethanolamine (DOPE) was mixed in an optimal concentration (15 nmol DC-Chol/Dope) and combined with 1 $\mu$g DNA in 0.7 mL (5,6). In the second trial, the DNA/liposome formulation is prepared by incubation of DNA (5 $\mu$g) and dimyristoyloxy-propyl-3-dimethyloxyethyl ammonium (DMRIE)/DOPE (15 nmol) in a final volume of 1 mL in lactated Ringers solution (7).

### 3. Methods

#### 3.1. Study Design for DC-Cholesterol/DOPE Gene Transfer (Clinical Protocol I)

Patients with stage IV melanoma who met guidelines of the clinical protocol (8) are treated in the Clinical Research Center. Informed written consent is obtained according to The Committee to Review Grants for Clinical Research and Investigation Involving Human Beings, the Recombinant DNA Advisory Committee of the National Institutes of Health, and the Food and Drug Administration.

A cutaneous tumor nodule is identified for treatment, and its borders measured. Other control (untreated) nodules are quantitated by computerized tomography immediately prior to the procedure. For our first clinical protocol, the DC-Chol/DOPE liposome preparation was used with the indicated DNA dose escalation. At each treatment, Group I ($n = 3$) received 1 injection of $0.2 \text{ mL}$ of DNA liposome (0.29 $\mu$g DNA) complex into the tumor (0.86 $\mu$g cumulative dose). Group II ($n = 3$) received 3 injections (0.2 mL each) at one session within the same nodule (2.58 $\mu$g cumulative dose). All patients receive a total of three treatments with a 2-wk interval between treatments.

#### 3.2. Preparation and Administration of DNA Liposome Complex

DNA liposome complexes are mixed immediately prior to injection by adding 0.1 mL of lactated Ringer’s solution into a sterile vial of HLA-B7 plasmid DNA (20 $\mu$g/mL; 0.1 mL). An aliquot of this solution (0.1 mL) is added at room temperature to 0.1 mL of 150 $\mu$M (dioleoyl phosphatidylethanolamine/3b [N-($N'$,$N''$-dimethylaminoethane)-carbamoyl] cholesterol) liposome (5) in
lactated Ringer’s solution in a separate sterile vial. The DNA and liposome vials are prepared in accordance with Food and Drug Administration guidelines and quality control procedures. After incubation for 15 min at room temperature, an additional 0.5 mL of sterile lactated Ringer’s solution is added to the vial and mixed. The DNA liposome solution (0.2 mL) is injected into the patient’s melanoma nodule under sterile conditions at the bedside after administration of local anesthesia (1% lidocaine), using a 22-gage needle. The injection is performed once per treatment session (Group I, patients 1, 2, 3A) or three times per treatment session (Group II, patients 3B, 4, 5). For catheter delivery, the DNA liposome solution (0.6 mL) is delivered into the artery using percutaneous delivery.

For pulmonary artery gene transfer, percutaneous right heart catheterization is performed from the right femoral vein using sterile techniques. An 8-French sheath was inserted into a right femoral vein, and a 7-French Van Aman pigtail catheter is advanced into a pulmonary artery under fluoroscopic guidance. After pressure measurement, selective catheterization of an artery is performed, and the catheter is exchanged for a 5-French occlusion balloon catheter (Medi-Tech, Watertown, MA) over a 0.025-in. exchange guidewire. After balloon inflation, digital subtraction angiography is performed to confirm catheter position and arrest of blood flow. Confirmation that blood flow is arrested is performed by inflation of the balloon injection of iv contrast dye into the vascular space. No diffusion of the contrast material should be noted over a 5-min period, thus ensuring that delivery of DNA and liposomes proceeds antegrade in the vasculature and is not immediately admixed with blood to inactivate the DNA liposome complex. The artery is rinsed with 30 mL of sterile saline, and the HLA-B7 DNA liposome solution, 1.43 μg of DNA in a volume of 0.6 mL, is instilled, followed by an additional 1.0 mL of sterile saline. This solution is injected through the end hole of the catheter into the artery. An additional 3.0 mL of sterile saline is instilled through the catheter, and the HLA-B7 DNA liposome solution incubated for 20 min to achieve transduction of the local microcirculation. The balloon is deflated, and the catheter and femoral sheath are removed. To determine the effects of DNA liposome delivery to the pulmonary vasculature, hemodynamic and biochemical monitoring is performed. The patient’s electrocardiogram (ECG) (six-lead) is continuously recorded prior to, during, and after injection of the DNA liposome solution. Pulmonary artery, pulmonary capillary wedge pressure, and systemic blood pressure are measured 5 min prior to gene transfer and 20 min after delivery of DNA and liposomes.

Biochemical parameters are also measured one day before and one day after each treatment. During each treatment, 0.6 mL of DNA liposome solution are administered followed by an additional total of 4 mL of sterile saline to distribute it to the local microcirculation.
3.3. Analysis of HLA-B7 Gene Expression

To confirm recombinant HLA-B7 gene expression within treated tumor nodules, core needle biopsy samples of the injected tumor are performed 3–7 d after gene transfer. Genomic DNA is isolated from biopsy material (3), and PCR for HLA-B7 gene is performed with primers: (sense) 5' AGT GCC CAG GGC TCT GAT GTG TCT CTC ACA 3' (HLA-B7) and (antisense) 5' ACC ACA GAA GTA AGG TTC CTT CAC AAA GAT 3' (SV40 poly A), yielding a 301-bp fragment. For the RNA analysis, the same primers are amplified after reverse transcription with an oligo dT primer. Analysis of plasmid in blood is performed with the same primers or a different set: (sense), 5' CTA CGT GGA CGA CAC CCA GTT CGT C 3' and (antisense) 5' AGG GTG GCC TCA TGG TCA GAG ATG G 3', yielding a 525-bp fragment. RNA in analyzed by PCR after DNase digestion and incubation with reverse transcriptase as described (9). Southern blot hybridization of PCR products from the DNA and RNA analysis in performed with an internal probe from pHLA-B7 derived by digestion with PvuII and BglII by standard methods (10). A control using 293 cells transfected with plasmids in vitro is used to define conditions to establish the conditions for DNase digestion. Typically, DNase (225 U/reaction) is used to digest DNA in a total volume of 20 µL. Under these conditions, no PCR signal should be detected in the absence of reverse transcription.

3.4. Study Design for DMRIE/DOPE Gene Transfer
(Clinical Protocol II)

In patients undergoing this gene transfer protocol, the tumor nodule and its borders are measured prior to injection. A needle biopsy is performed when possible to confirm the diagnosis and for analysis as a pretreatment sample. Alternately, a separate untreated nodule is used. Tissue is stored as frozen sections for further immunohistochemical analysis and PCR. In addition, this nodule and other control (untreated) nodules are imaged by CT immediately prior to the procedure, and its size quantitated. The skin overlying the tumor nodule is sterilized and anesthetized using 0.01% lidocaine. For gene transfer, a 22-gage needle is used to inject the DNA liposome complex. The complex is prepared as follows: 10 min prior to delivery, 0.1 mL of plasmid DNA (0.05–50 mg/mL) in lactated Ringer's solution is added to 0.1 mL of DMRIE/DOPE liposome solution (0.15–15 µM). Each component is stored separately in sterile vials and certified as acceptable by the FDA. The solution is left at room temperature for 5–10 min and 0.8 mL of sterile lactated Ringer's is added to the liposome DNA solution. The optimal composition of the DNA/liposome complex has been established for each batch by titration of DNA concentration and liposome concentration independently on human melanoma and renal cell
carcinoma in vitro, and confirmed by direct injection into melanoma or other tumors in vivo prior to use. Each component, the liposome preparation and the DNA, is tested for contaminants and toxicity and used according to previously established guidelines. The liposome solution and DNA is aliquoted in individual sterile vials mixed under sterile conditions.

For direct injections of the HLA-B7 plasmids, escalating doses are studied. Four groups (three patients each) are studied sequentially with at least 1 mo of observation prior to evaluation of the next group. Patients in each group receive intratumor injections. Group I receives 3 injections of 0.2 mL within the same nodule (3 µg of DNA plus 4.5 nM DMRIE/DOPE). Group II receives the same treatment with a 10-fold higher concentration of DNA liposome complex. Group III receives 100-fold higher dose, and Group IV receives 1000-fold higher amount.

For catheter-based gene delivery, the same dose escalation is used, except a single 0.6-mL injection into the end artery that perfused an isolated nodule is used with an occlusion balloon catheter. Doses are repeated within each subject for whom the toxicity treatment is with grade II. Dose escalation begins if three patients show toxicities < grade III from the treatment. If one patient displays toxicity > grade II, the treatment is repeated on three additional patients. If two patients develop toxicity > grade II, the dosage is reduced. The maximal tolerated dose is defined as the dose at which two or more patients out of six develop grade III or IV toxicity. Once the treatment dose is defined, an additional 4–6 patients are entered at that dose to ascertain the safety of this dose for wider application.

Prior to the injection with the needle in place, gentle aspiration is applied to the syringe to ensure that no material is injected iv. Immediately after the injection procedure, a blood sample is obtained to check serum enzymes, chemistries, and blood counts, and to analyze for the presence of plasmid DNA in the peripheral blood by PCR. The patient is observed in the Clinical Research Center for an additional 24–48 h, and another blood collection is performed after this time. If there are no complications, the patient is discharged after 24–48 h.

3.5. Confirmation of Gene Transfer and Expression

Needle biopsy of the injected nodule is performed after administration of local anesthesia prior to injection and subsequently to treatment (preferably at d 3). If sufficient material can be obtained, RNA PCR analysis is performed. A portion of this tissue is processed to obtain DNA for PCR analysis. The remaining tissue is processed for pathologic analysis and immunohistochemical and/or immunofluorescent staining. For internal organs, CT- or ultrasound-guided thin needle biopsies will be obtained when possible.
3.6. Analysis of Immune Response

Evidence of gene transfer is obtained indirectly by examination of the specific immune response to HLA-B7. The analysis is performed as follows: two weeks prior to the initial treatment, a blood sample is obtained to derive lymphocytes that will be immortalized using the Epstein–Barr virus. An aliquot of these cells is further infected with an amphotropic HLA-B7 retroviral vector, and expression is confirmed on the cell surface. These cells are subsequently used in the laboratory as target cells for the cytolytic T-cell assay.

References
Methods for Retrovirus-Mediated Gene Transfer to Tumor Cells

Elizabeth M. Jaffee

1. Introduction

The past several years have seen a renewed interest in active immunotherapy approaches for the treatment of human tumors because of the exciting findings learned from preclinical studies employing genetically altered tumor vaccines. Many clinical protocols are currently being conducted using several different genetic approaches for augmenting antitumor immune responses. These include:

1. Ex vivo gene transfer of cytokine genes into tumor cells.
2. Ex vivo gene transfer of suicide genes into tumor cells with subsequent activation of the suicide mechanism in vivo.
3. Ex vivo cytokine gene transfer to cultured tumor-infiltrating lymphocytes (TIL) to generate more potent T-cells for adoptive transfer.
4. In vivo gene transfer of major histocompatibility complex (MHC) class I molecules by direct injection of the gene into an accessible tumor mass.

Whereas the recent preclinical studies performed in mouse tumor models are indeed paving the way for clinical trials with genetically engineered tumor vaccines, a number of critical technical considerations remain that will have significant impact on whether or not this new immunotherapy approach will translate into true clinical benefit for human cancer patients. First, most human tumors are difficult to establish as cell lines, even in the short-term. Yet, until cellular tumor antigens are identified, autologous cell lines will need to be established from each patient to be treated, as a means of providing tumor antigens at the site of activation of the immune system. Even in cases where subselection of continuously growing tumor lines can be achieved, there is a high likelihood that, after extended passage, the antigenic composition will change significantly, relative to the original primary tumor from which the
tumor line originated. Second, high efficiency gene transfer systems are needed to genetically alter these primary tumor lines. Ideally, high-efficiency gene transfer would obviate the need for cotransduction of a selection marker, thereby minimizing the level of in vitro passage and maximally preserving the original antigenic composition of the explanted primary tumor.

Defective retroviral vector systems are unquestionably the gold standard for high-efficiency gene transfer in the initial stages of human gene therapy. These vectors have the potential for being free of helper virus, and are therefore extremely safe. Nonetheless, they require at least one cell cycle in order for integration and gene expression to occur. Early generations of retroviral vectors have been notoriously inconsistent in their gene transfer efficiency as well as in consistency of gene expression. Recently, newer generations of retroviral vectors that have taken advantage of modifications in the intronic gag sequences as well as positioning of the start sites of the inserted gene, appear to have successfully circumvented a number of these problems and allowed efficient gene transfer into primary human tumor explants in the absence of selection (1). Other gene-transfer systems such as adenovirus-based systems as well as nonviral gene transfer systems including liposomes, are currently under evaluation, though their ultimate usefulness in clinical cancer settings remains to be determined. These alternative systems are discussed in other chapters.

This chapter will provide an overview of retrovirus-mediated gene transfer as it relates to cancer therapy, and a detailed discussion of the methods required for ex vivo, retrovirus-mediated gene transfer to tumor cells.

1.1. Rationale for the Use of Genetically Altered Tumor Cells for the Treatment of Cancer

1.1.1. The Role of the Immune System in the Treatment of Cancer

The rationale for genetically altered, tumor vaccine-based immunotherapy of established cancer depends on the existence of antigens within tumors that can be recognized as foreign by the host immune response. It is the tremendous diversity of the T- and B-cell receptors that endows the immune system with the ability to distinguish fine antigenic differences among cells. For the generation of an antitumor immune response, two criteria must be fulfilled. First, the tumor must present novel antigens or neoepitopes not found on normal cells. Second, the immune system must be appropriately activated to respond to these novel antigens. The classic studies of Boon and colleagues (2–3) have demonstrated that tumor antigens recognized by T-cells fall into one of three categories:

1. Novel peptide sequences generated by point mutations in genes encoding various cellular proteins
2. Examples in which the gene encoding the tumor antigen is identical to the germ-line sequence, but is not expressed in any normal tissues.

3. Examples in which the gene encoding the tumor antigen is a highly specific differentiation antigen.

As a consequence, the immune system need not be tolerized to the gene during development, and peptides derived from the nonmutated form can serve as tumor-specific antigens. The MAGE-1 gene encodes a human melanoma antigen that was the first human antigen identified. The MAGE-1 antigen falls into the second category of tumor antigens, and is expressed in as many as 50–60% of human melanomas (3). The tyrosinase protein is an example of a differentiation antigen that encodes for another human melanoma antigen (4). This antigen was found to be expressed by 39 of 39 fresh melanoma samples tested, and was also overexpressed relative to normal skin samples. These antigens, plus an additional set of differentiation antigens more recently identified in human melanomas (5), have indeed renewed faith in the concept of common sets of tumor-specific antigens, thereby allowing for the possibility of specific antigen or peptide-based vaccination strategies. But until more common tumor antigens have been identified at the genetic level, and the prevalence and biorelevance of these antigens has been assessed, an individual’s tumor is the only source of tumor antigens for vaccination at this time. An intensified search by many groups of investigators to identify other tumor antigens expressed by tumors other than melanoma is currently underway.

1.1.2. History of the Development of Gene-Altered Tumor Vaccines

The first studies demonstrating enhanced immunogenicity of genetically altered tumors were performed 25 yr ago, starting with Lindenmann and Klein (6), who showed that vaccination with influenza virus-infected tumor cell lysates generated enhanced systemic immune responses against a challenge with the original wild-type tumor cells. Furthermore, these early studies showed that nonvirally infected tumor cell lysates, or tumor cell lysates mixed with the same virus, are not immunogenic, and cannot elicit a systemic immune response against challenge with the parental tumor cells. Because adequate immunization against the tumor required that the tumor cells be infected with the virus, Lindenman and his colleagues hypothesized that weak antigens derived from the tumor cells might become associated with or incorporated into the virus, and subsequently become potent immunogens. However, based on what we have learned since then about immune responses, the enhanced immune response generated by the virally-infected tumor cells was probably the result of high viral protein expression and subsequent availability of both MHC class I and II antigenic epitopes required for priming the CD4+ and
CD8+ T-cell arms of the immune system. The antitumor response that was generated in addition to the antiviral response, probably occurred as a consequence of the expression of tumor antigens at the same site as the viral antigens. It is important to point out that the studies of Lindenmann and his colleagues provide the first evidence that employing immune modulators in a paracrine fashion, may be a critical first step for generating effective, specific, antitumor immune responses. As newer techniques of gene transfer have been developed, infection with infectious virus has been replaced with specific gene transfer in an attempt to more carefully regulate the nature of the genetic alteration in the tumor.

This and other studies have encouraged an increased effort in murine tumor models aimed at directly altering the tumor cell's genetic material to enhance immune responses generated against them. The enhancement of immune responses in the vaccine setting can be divided mechanistically into two categories. First is enhancement of the presentation of antigens to T-cells. This mechanism implies an increase in peptide-MHC density at the site of activation of T-cells. Second is enhancement of costimulation. This mechanism takes into account the fact that T-cells require extra costimulatory signals in addition to engagement of the T-cell receptor in order to become efficiently activated. Costimulatory signals may either be membrane-bound ligands such as B7 that are critical for T-cell activation, or soluble mediators such as cytokines. Because so many of the molecules necessary for antigen presentation and costimulation have been identified and cloned, it was natural to utilize gene transfer strategies to alter the expression of these molecules locally.

1.1.3. Murine Studies Employing Genetically Altered Tumor Vaccines

Introduction of MHC genes into tumors was among the first approaches for enhancing the immunogenicity of tumors. Increasing MHC class I expression by gene transfection typically results in decreased tumorigenic capacity in murine tumor models (7). The decreased tumorigenicity is felt to be owing to enhanced presentation of tumor-specific antigens to CD8+ CTLs in vivo. However, enhanced expression of self MHC class I molecules does not always increase immunological potency of a tumor and, in certain circumstances, has been shown to inhibit natural killer (NK) cells, thereby resulting in paradoxically increased tumorigenicity (8). Tumor immunogenicity has also been enhanced by the transfer of allogeneic MHC class I genes (9,10). In certain cases, the rejection of tumors expressing allogeneic MHC class I molecules may result in enhanced systemic immune responses against subsequent challenge with the unmodified parental tumor. This represents an example of the general phenomenon of enhancing tumor vaccine potency by introducing genes encoding any foreign antigen (referred to as xenogenization). The mechanism
by which xenografted tumors enhance systemic immune responses against
challenge with the parental tumor is still not clear, although the mechanism has
been postulated to be the result of nonspecific effects owing to increased local
cytokine induction.

There has been more interest in the study of immune responses generated by
tumor cells genetically engineered to secrete various cytokines. This strategy
does not involve inducing the expression of any foreign genes in tumor cells,
but rather seeks to locally alter the immunological environment of the tumor
cell so as to either enhance antigen presentation of tumor-specific antigens to
the immune system or to enhance the activation of tumor-specific lympho-
cytes. As mentioned, one of the most important concepts underlying the use of
cytokine gene-transduced tumor cells is that the cytokine is produced at very
high concentrations local to the tumor. Systemic concentrations are generally
quite low. This paracrine physiology much more closely mimics the natural
biology of cytokine action than does the systemic (iv) administration of recom-
binant cytokines. Many cytokine genes have been introduced into tumor cells
with varying effects on both tumorigenicity and immunogenicity. Some of
these cytokines, when produced by tumors, induce a local inflammatory
response that results in elimination of the injected tumor. This local inflamma-
tory response is most often predominately dependent on leukocytes other
than classical T-cells. These systems have been used to uncover in vivo effects
of cytokines that result in activation of tumoricidal potential by various types
of leukocytes.

This new tumor vaccine approach, using genetically-altered autologous
tumor cells to secrete local concentrations of cytokines, has been best devel-
oped in murine models (11–19). In some of these models, lymphokine gene-
transduced tumor cells have been shown to generate a local, tumor-specific,
immune response when administered as a subcutaneous vaccination (11–
13,15–19). In addition to rejecting the genetically modified tumor cells, vacci-
nated animals may develop a T-cell-dependent systemic immunity that, in
some cases, can cure micro metastases established prior to treatment with the
genetically altered tumor cells (11–13,17,18). In all cases in which systemic
immunity against wild-type tumor challenge has been analyzed, it is mediated
by T-cells. However, given the number of studies done to date with cytokine-
transduced tumor cells, it is not surprising that variable results have been seen
when different tumor systems are analyzed. Additional variables to the
cytokines employed include: cell dose, level of cytokine expression, location
of immunization and challenge sites, and vaccination schedule.

Given the large number of potential cytokine genes in the armamentarium,
and the technical difficulties in transducing human tumor cells to make vac-
cines, it is critical that they be compared for efficacy. Also, given that most
mouse tumors show significant immunogenicity when simply irradiated, identification of genes that truly enhance a tumor's immunogenicity above that of irradiated wild-type cells is important. The first study that directly compared multiple cytokine and other genes in murine tumor models used a highly transmissible, defective retroviral vector. This study demonstrated that, in a number of poorly and moderately immunogenic tumors, including a murine renal cell carcinoma, immunization with granulocyte-macrophage colony-stimulating factor (GM-CSF)-transduced tumors produced the greatest degree of systemic immunity, which was enhanced relative to irradiated nontransduced tumors (17). Immunity was dependent on both CD4+ and CD8+ T-cells, despite the fact that the tumors were MHC class I+. The potency of GM-CSF's effect locally may relate to its unique ability in promoting the differentiation of hematopoietic precursors to dendritic cells, which are the most potent antigen-presenting cells for helper T-cells (20,21).

1.2. Features of Retroviral Vectors

1.2.1. Rationale for Choosing a Retroviral Vector for Gene Transfer into Tumor Cells

Gene transfer into tumor cells can be accomplished by a variety of methods involving either naked DNA or the use of viral vectors. There are many methods for transferring naked DNA into cells, including:

1. Coprecipitation with calcium phosphate (22).
2. The use of electroporation that exposes cells to rapid pulses of high voltage current, thereby providing a physically induced opening in the cell membrane for entry of DNA (23).
3. Direct introduction of DNA into cells by microinjection (24)
4. Encapsulation of DNA into liposomes (25).

Use of any of these methods will often result in the introduction of multiple copies of the cytokine gene randomly into the host cell's genome. Several of these methods can result in transient expression of the gene (for 24–72 h) by as many as 50% of the cells in the transfected population, because the transfected DNA can exist free in the cell nucleus for a short time (26). However, stable gene expression that requires integration of the transfected DNA into the host's genome usually occurs in <1% of the cells within the population undergoing transfection. To achieve stable integration and expression of the DNA into a high proportion of the cell population, it is often necessary to select for the minority of cells in the transfected population that have successfully retained the foreign DNA. This can be accomplished by cotransfecting DNA that encodes for a selectable marker, that will allow cells expressing its product to survive in growth media that contains a substrate for the gene's product, that is
normally toxic to most mammalian cells. In this way, in vitro selection of those cells that have successfully incorporated the transferred DNA can be accomplished. However, although in vitro selection will enhance the number of gene-transduced tumor cells in the cell population to nearly 100%, it is at the theoretical expense of antigen expression loss among that tumor cell population. In theory, loss of particular antigenic populations of tumor cells will decrease the effectiveness of the vaccine.

Currently, the most efficient method of stable gene delivery into mammalian cells is through the use of viral vectors, which infect their target cell by binding specific cell surface receptors. Most viral vectors are constructed so that they contain the sequences encoding for the expression of the gene and all of the genetic signals including the promoters, enhancers, splicing signals, and signals for polyadenylation of RNA transcripts, all of which are necessary for the transcription and ultimate translation of the inserted cytokine gene sequences. Often, the vectors will also contain selectable markers (27). Potential adverse consequences following viral infection can include:

1. Damage or death to the host cell.
2. The activation of other latent viruses integrated into the host’s genome.
3. The activation of silent host genes such as proto-oncogenes.
4. Transformation of the defective viral vector from replication-incompetent to replication-competent by recombination with host gene sequences (which results in the production of helper virus).

All of these possibilities should be considered in choosing a viral vector system for gene transfer.

Retroviruses have been the most commonly employed vectors for the preparation of cytokine-secreting tumor vaccines, for study in murine models, and in human vaccine therapy trials. There are at least two reasons for this. First, as mentioned above, retroviruses usually do not enter into a lytic cycle of viral replication and therefore, do not kill their host cell soon after viral infection. Second, retroviruses can infect most mammalian cells and integrate into the host genome, which is a critical requirement for efficient gene transfer and expression in a stable and heritable fashion. Most of the retroviral vectors employed in cytokine-secreting tumor vaccine studies have been developed from either avian or murine retroviruses. A key feature of these retroviral vectors is their incompetence to replicate following transduction into the host cell. Details of the mechanisms of infection, replication, integration, and gene expression of these viral vectors have already been described in significant detail in the literature (28–33). However, it is important to note that host cell replication and DNA synthesis are required for provirus integration, and thus, efficient gene transfer is restricted to replicating cells.
1.2.2. Structure of the Retroviral Vector

The transduction procedures that will be described employ the MFG retroviral vector system. However, several other retroviral vectors are also available for this use (34,35). The structure of MFG has recently been described (20,33). Briefly, in this vector, Moloney murine leukemia virus (Mo-MuLV) long-terminal repeat (LTR) sequences are used to generate both a full-length viral RNA (for encapsidation into virus particles) and a subgenomic mRNA (analogous to the Mo-MuLV env mRNA) that is responsible for the expression of inserted sequences. The vector retains both sequences in the viral gag region shown to improve the encapsidation of viral RNA (20) and the normal 5' and 3' splice sites necessary for the generation of the env mRNA. Protein coding sequences are inserted between the NcoI and BamHI sites in such a way that the cDNA sequences encoding the gene of interest are cloned into the downstream site such that the cDNA inserts AUG at the exact position relative to the 3' splice acceptor site where the env gene starts in the original virus. It is therefore expressed as a subgenomic transcript off of the 5' LTR. No selectable marker exists in the vector. This feature, together with deletion of sequences in the 5' portion of the gag-region intron, results in high titer production of viral particles by the packaging line as well as uniformly high levels of expression regardless of the particular gene cloned into the vector.

The use of these high efficiency retroviral vectors to carry cytokine genes into tumor cells has a number of specific advantages:

1. The transduction is rapid and selection is unnecessary. Subcloning experiments documented that nearly 100% efficiency is achieved in a 4-h infection followed by 48 h culture to allow for integration of the viral vector into the host cell's genome.

2. The absence of selection avoids the potential problem that selected subclones may display different immunologic and biologic properties from the original tumor cell population.

3. Using retroviral supernatants from different cytokine producer cell lines, combinations of cytokines can easily be simultaneously introduced into the same cell.

However, there are also several potential disadvantages to the use of this vector system. First, entry into the cell is dependent on the expression of specific viral receptors on the host's cell surface. Because the identity of most retroviral receptors is still unknown, it is difficult to determine whether or not a particular host cell expresses these receptors, and, to what extent these receptors are expressed. As will be discussed, the retroviral packaging lines do have the ability to produce virus particles that can infect a wide host range. Second, as mentioned earlier, efficient retroviral gene transfer requires that the host cell proliferate for integration of the viral genes into the host
cell’s genome, and for expression of the transferred gene. Third, retroviruses are difficult to purify, concentrate, or store for long periods of time, because of their fragility. Their fragility is probably manifested by the loss of their env gene product, which ultimately translates into decreased infectivity. Fourth, there is the theoretical concern that retroviral integration may lead to insertional mutagenesis because the virus integrates into random sites in the host’s genome, and may interrupt vital cellular genes or insert retroviral regulatory sequences that modify the expression of nearby cellular genes. Finally, as will be discussed in Section 1.2.3., helper virus production by the retroviral packaging line has occurred when employing some of the earlier generations of these packaging lines. This potential problem is the major concern when choosing a viral vector to employ for the production of genetically altered tumor vaccines for clinical trials. Helper virus detection has not been reported to have occurred in any of the clinical trials employing retroviral vectors thus far, nor in most of the animal studies designed to test the safety of these vectors. However, in one study in which immunosuppressed monkeys purposefully received high concentrations of replication-competent retroviruses, lymphomas developed (36), illustrating the potential seriousness of helper virus contamination.

1.2.3. Construction of the Packaging and the Producer Lines

The other critical component of the retroviral vector system is a cell line that produces the viral proteins that are required for encapsidation of the viral RNA. This cell line, referred to as the retrovirus packaging line, is produced by transfection of proviral DNA containing the retroviral genes necessary for the synthesis of the viral proteins, into a fibroblast cell line. The most commonly employed fibroblast cell line is the murine NIH 3T3 cell line. After transfection of the proviral DNA vector into the packaging cell line, this line is then referred to as the retrovirus producer cell line. The first generation of packaging lines contained the stable introduction of a mutant Moloney murine leukemia virus proviral genome that contained a deletion of the encapsidation sequence (psi sequence) (30,37). However, these packaging cell lines have been shown to produce low levels of replication competent virus, both psi negative, and wild-type virus, through recombinational events involving a copackaged recombinant genome carrying the psi sequence, at low efficiency (38). Newer constructs of packaging lines have not been shown to produce helper virus (29,31,39,40). Most of the producer cell line clones derived from these packaging cell lines have been shown to produce retroviral titers in the range of $10^6$ cfu/mL, levels that allow for high efficiency gene transfer (29). These titers are comparable to low normal titers obtained for replication-competent retroviruses.
2. Materials

1. Retroviral vector producer lines: The MFG retroviral producer cell lines were obtained from R. C. Mulligan (Whitehead Institute for Biomedical Research, Cambridge, MA). Both the amphotropic and ecotropic retroviral producer cell lines, CRIP and CRE, respectively, are grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with high glucose (4500 g/L), supplemented with 10% bovine calf serum, penicillin (100 U/mL final concentration), streptomycin (100 µg/mL final concentration), L-glutamine (2 mM final concentration), and gentamycin (50 µg/mL final concentration), at 37°C, and 10% CO2. trypsin (0.25%)-EDTA (0.1%) for passaging the cell lines.

2. Tumor cell lines. All tumor cell lines to be transduced should be maintained in their optimal growth media before and after performing the transduction procedure to enhance the proliferation capacity of the cell population.

3. Retroviral gene transfer to tumor cells: Tumor cells and retroviral supernatant that has been prepared as described in Methods. In addition, DEAE-dextran 10 mg/mL stock solution prepared by dissolving 1 g into 100 mL of the producer line growth media (DMEM + 10% calf serum), and filtered through a 0.45-µm filter. Store in sterile 5-mL aliquots at 4°C for up to 6 mo. Sterile tumor growth media and sterile 1X PBS.

3. Methods

3.1. Maintenance of Retroviral Vector Producer Lines

1. Grow the retroviral producer cell line in culture to confluency in large flasks (162-cm² or greater). These cells grow in an adherent monolayer.

2. When the cells have reached confluency, remove the supernatant, and incubate the cells with enough trypsin-EDTA to cover the bottom of the flask (usually 2–3 mL) at room temperature until the cells become nonadherent (usually 1–2 min).

3. Quench the trypsin with at least 3–4 vol of the growth media containing the calf serum.

4. Centrifuge for 10 min at 1500 rpm, at 4°C.

5. Remove the supernatant and count the cells.

6. Replate the cells at about a 1:10 dilution of the number of cells in the confluent flask (about 2 × 10⁶ cells per 162-cm² flask).

7. Split the cell lines 1:10 every 3–4 d, or when each flask reaches confluency.

3.2. Preparation of Retroviral Supernatants

1. Two days prior to transduction, trypsinize the producer cells, wash them once, and replate them at a density of 2 × 10⁶ cells per 100-mm culture dish.

2. One day prior to transduction, remove the media, and add 10 cc of fresh media to the cells.

3. On the day of transduction, collect a 24-h retroviral supernatant and filter through a 0.45-µm filter to remove contaminating retroviral producer cells.
3.3. Maintenance of Tumor Cell Lines

1. Grow the tumor cells in culture in optimal tumor growth media to confluency.
2. On the day prior to transduction, replate the cells at a density of \(2–5 \times 10^5\) cells per 75-cm\(^2\) culture dish.

3.4. Performing Retroviral Gene Transfer to Tumor Cells

1. Incubate the freshly collected retroviral supernatant with the 10 \(\mu\)g/mL final concentration of the transduction enhancer DEAE-dextran (see Section 4.4.) for approx10 min at room temperature, so that the retrovirus will bind to the enhancer prior to exposure to the tumor cells.
2. Remove the growth media from the tumor cells and replace it with 10 cc of retroviral supernatant containing the enhancer.
3. Incubate the cells at 37°C for 24 h (see Section 4.4.)
4. Following incubation of the tumor cells with the retroviral supernatant, remove the supernatant, and wash the cells twice with sterile PBS to rinse away residual retroviral supernatant.
5. Add 10 cc of tumor growth media and allow the cells to grow for 48 h.

3.5. Testing for Cytokine Gene Product

1. At 48 h following transduction, remove the growth media, and add 10 cc of fresh tumor growth media.
2. Collect a 24-h supernatant for evaluation of cytokine secretion. To do this, remove the supernatant, centrifuge or filter through a 0.45-\(\mu\)m filter to remove the cells, and aliquot the supernatant into three 1-mL, sterile aliquots that can be stored frozen at \(-70^\circ\text{C}\) until the time of testing for cytokine secretion. Three separate aliquots should be stored so that repeat testing can be performed without multiple freeze/thaws (which might reduce the concentration of the gene product).
3. Following collection of the cell supernatant, take up the cells and count them. Record the total number of cells that contributed to the production of the cytokine over 24 h. This number will be used to calculate the concentration of cytokine secretion per given number of tumor cells after the concentration of cytokine in the supernatant is determined.

4. Notes

4.1. Maintenance of Retroviral Vector Producer Lines

1. Producer lines derived from the NIH 3T3 fibroblast cell line grow in an adherent monolayer. They do best when they are plated at a threshold density of about 1/10 the flask’s total cell capacity. Plating the cells at a lower density may result in loss of the cell line.
2. Exposure to trypsin results in the rapid release of the producer cells from the tissue culture flask. Be aware that overexposure to trypsin will result in significant cell death.
3. The MFG producer line grows in media supplemented with bovine calf serum. Substitution of fetal bovine serum may result in a change in growth kinetics and viral particle production. The growth requirements recommended by the laboratory in which the producer line originated should always be used to culture the producer line being employed.

4. It is advantageous to initially expand enough of the producer cells to allow for freezing down of a large stock of aliquots for two reasons. First, prolonged passage in culture may increase the possibility of recombination events within the producer cells that may result in the production of helper virus. Second, there is the theoretical concern that prolonged passage in culture may result in a decrease in the population of producer cells capable of efficiently producing the viral particles. The MFG producer cell lines freeze well in 90% calf serum plus 10% DMSO. Recovery of viable producer cells will be severely compromised if these cells are frozen in other types of serum. Each producer line should be frozen in the same type of serum that is used for in vitro growth. These cells can be stored long-term in liquid nitrogen.

4.2. Preparation of Retroviral Supernatants

6. Retroviruses are difficult to titer because they do not form plaques. It is therefore recommended that every retroviral producer line be titered for transduction efficiency using an easily transducible cell line. NIH 3T3 cells are a good choice for comparison with other murine cell lines. For the transduction of human primary cultures, a human cell line may be a more appropriate cell line for comparison. Titering can be accomplished by using the transduction procedure described in Sections 3.1.-3.5., and by performing serial two- to five-fold dilutions of the retroviral supernatant prior to exposure of retrovirus to the cell line. Dilutions of the retroviral supernatant should be made with the cell line’s growth media for best results. Most supernatants are optimal either undiluted, or between a 1:2 and 1:10 dilution.

7. Before assuming that insufficient transduction rates are owing to low titer supernatants as a result of a poor supernatant collection, it is important to first determine if the producer cells themselves are still capable of producing high quantities of retroviral particles. Because the producer cells themselves also express the gene encoded by the retroviral vector, an easy way to evaluate the producer line for production of the vector is to assay the cells for expression of the gene product. However, in vitro loss of high titer producer lines owing to long-term culture can easily be avoided by routinely thawing a new aliquot of producer cells every 3-4 wk.

8. If expression is at the expected level, then the problem is more likely to be owing to a low titer retroviral supernatant resulting from suboptimal supernatant collection. There are two major causes of low titer retroviral supernatants: inadequate retroviral supernatant collection resulting from insufficient numbers of producer cells or overgrowth of producer cells; and suboptimal growth conditions for retroviral supernatant collection, in particular, a bad lot of calf serum, use of the wrong media and supplements, inadequate CO₂ concentration during incubation, and so on.
9. A recent study performed to evaluate the improvement of retroviral vector production observed that the growth of 21/22 producer cell lines at 32°C for up to 2 wk after the cells reached 100% confluence increased vector titers (42). Growth of the producer lines at 32°C is thought to increase the stability of the viral particles. In addition, improved vector production may be a result of the decreased metabolism of the producer cells at this lower temperature.

10. For optimal transduction efficiencies, freshly collected retroviral supernatants should be used. Although it is possible to store the supernatants at 4°C for several days, and to freeze these supernatants at −70°C for several weeks, the efficiency of transduction may decrease by as much as 50% following thaw of the supernatant.

11. Producer lines must be frozen in the same type of serum used for in vitro growth unless otherwise advised. The substitution of other serum may not support the growth of these cells well, and may result in significant cell death during freezing and storage.

4.3. Maintenance of Tumor Cell Lines

12. Most proliferating cell lines can be transduced with a retroviral vector. However, the efficiency of transduction will depend on the percentage of cells that are actively proliferating at the time of exposure to the retrovirus, since integration into the host genome is required for stable expression of the transferred gene. Therefore, the growth conditions for each cell line being transduced should be optimized before attempting this transduction procedure. Most long-term cell lines already have defined growth conditions that support optimal growth. However, it is now possible to transduce many primary, short-term cancer cell lines, and conditions for optimizing their growth may already have been described.

13. The actual density of cells in the flask should be optimized for every tumor cell type, keeping in mind that the cells will need to be able to proliferate maximally for at least 48 h following transduction to allow for optimal integration and expression of the gene. For cells with a 48–72 h doubling time, adequate transduction can be achieved by plating the cells at a density that will result in approx 1/3 confluency of the flask on the day of transduction.

4.4. Performing Retroviral Gene Transfer to Tumor Cells

14. Both a negative and a positive control group should be included in each transduction experiment to confirm that gene expression is the result of gene transfer. A good negative control group is to incubate a flask of each cell type to be transduced with retroviral producer cell growth media containing the enhancing polymer alone. An adequate positive control group would include the transduction of any easily transducible cell line with the same lot of retroviral supernatant used to transduce the test tumor cells.

15. Transduction efficiency can be enhanced by the addition of polymers to the retroviral supernatant just prior to exposure of the target cells to the retroviral vector. Enhanced gene transfer is thought to occur via a charge-mediated mechanism that affects virus binding to or penetration of the target cell. The polycations
protamine, polybrene, and DEAE-dextran are routinely used for this purpose (1,33,43). In addition, liposome-forming compounds such as DOTAP (Boehringer Mannheim, Indianapolis, IN) have also been successfully used to enhance retroviral gene transfer and may be less toxic to the host cell than other enhancers (1). Liposome-forming agents probably enhance gene transfer into the host cell by first forming stable interactions with the virus, then adhering to the cell surface, followed by fusing with the cell membrane and releasing the virus into the cell cytoplasm (44). Because most enhancers are toxic to the cell lines at high concentrations, yet higher concentrations of polymers may be required for enhanced transduction efficiency to some cell lines, it is recommended that a titer of the enhancer be performed on each new batch of enhancers used, to determine the least toxic, most enhancing concentration of the polycation or lipid compound. Table 1 illustrates recommended ranges of polycation and lipid reagent concentrations for the commonly employed transduction enhancers.

16. Longer incubation times will increase the number of proliferating cells that are exposed to the retroviral vector, and therefore, may increase the efficiency of transduction. Hardy tumor cell lines may tolerate the retroviral supernatant containing low concentrations of enhancer for 24–48 h without significant cell death. However, primary human tumor cultures may not tolerate a change in the growth media for more than several hours. Therefore, it is best to perform a pilot study evaluating the rate of tumor cell death over time when exposed to the retroviral supernatant containing the enhancer, to optimize the transduction procedure.

17. There is recent evidence to suggest that the efficiency of retroviral transduction can be improved by a 90-min centrifugation at 2500 rpm, and 32°C, prior to an overnight incubation (at 32°C) of the tumor cells with the retroviral supernatant (42). However, some tumor cells may not tolerate an overnight incubation at 32°C.

18. It is often useful to perform the initial transduction studies on new tumor cell lines using the retroviral vector containing a marker gene (for example, the lacZ gene that expresses the cytoplasmic enzyme beta-galactosidase that will turn the transduced cell's cytoplasm blue when exposed to the substrate bluogal or X-gal). Marker genes can be used quantitatively to determine the number of tumor cells in the transduced population that are capable of expressing the transferred gene (the transduction efficiency of the vector for a particular tumor cell line).

19. It is not uncommon to have a high titer retroviral supernatant. If this is the case, the supernatant can be diluted 1:5 or 1:10 (depending on titer) with target cell growth media, prior to the transduction procedure, to decrease target cell toxicity from the retroviral supernatant. In fact, be aware that a dilution of a high titer retroviral supernatant may be necessary because higher titer supernatants may contain inhibitors against successful retroviral transduction.

20. The procedure described in Sections 3.1.–3.5. can be used for transduction of adherent and nonadherent tumor cell lines, and does not require any special modifications. For nonadherent cell lines, media changes require that the tumor cells be taken up with the supernatants and centrifuged to remove the supernatant. The cells are then resuspended in the fresh media and replated.
Table 1
Commonly Employed Transduction Enhancing Reagents

<table>
<thead>
<tr>
<th>Transduction enhancer</th>
<th>Target cell type</th>
<th>Concentration range (final concentration in retroviral supernatant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE-dextran</td>
<td>Murine tumor cell lines</td>
<td>5–10 µg/mL</td>
</tr>
<tr>
<td>(Sigma, St. Louis, MO)</td>
<td>Human tumor cell lines</td>
<td>10–100 µg/mL</td>
</tr>
<tr>
<td>Polybrene</td>
<td>Murine tumor cell lines</td>
<td>5–10 µg/mL</td>
</tr>
<tr>
<td>(Sigma, St. Louis, MO</td>
<td>Human tumor cell lines</td>
<td>10–100 µg/mL</td>
</tr>
<tr>
<td>or Aldrich Milwaukee, WI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>Murine tumor cell lines</td>
<td>5–10 µg/mL</td>
</tr>
<tr>
<td>(Lilly, Indianapolis, IN)</td>
<td>Human tumor cell lines</td>
<td>5–100 µg/mL</td>
</tr>
<tr>
<td>DOTAP</td>
<td>Murine tumor cell lines</td>
<td>5–10 µg/mL</td>
</tr>
<tr>
<td>(Boehringer Mannheim,</td>
<td>Human tumor cell lines</td>
<td>10–100 µg/mL</td>
</tr>
<tr>
<td>Indianapolis, IN)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

21. Freezing of large stocks of the transduced tumor cells is recommended to prevent loss of gene expression, as well as to prevent in vitro selection with loss of antigen expression. Transduced tumor cell lines can be frozen down and stored in liquid nitrogen long-term without loss of gene expression. Controlled-rate freezing is recommended to prevent a significant decrease in viability following thawing. A cheap and efficient way of control-rate freezing is to immerse the freezing vial of cells in a propanol bath (Nalgene Cryo 1°C Freezing Container), and to place the apparatus into a –70°C freezer overnight. This will freeze the cells at approx 1°C per min. The cells can then be placed into liquid nitrogen for long-term storage.

22. Primary human tumor lines are more difficult to transduce than long-term established lines. However, with the increasing applications of gene therapy to the clinics, there is an increasing need for improved methods of gene transfer to these cells. The most important criteria for efficient gene transfer to primary human tumor cultures is to optimize the growth conditions for maximal proliferation capacity. In addition, increasing the concentration of transduction enhancing polymer may result in improved transduction efficiency (1). It is often beneficial to initially screen the different enhancing polymers for the upper limits of concentration of polymer, and incubation time, that each primary tumor cell line can tolerate, before significant cell death is observed.

4.5. Testing for Gene Product Expression

23. Following transduction of tumor cells with the cytokine gene, the transduced cells should be evaluated for the total quantity of cytokine produced and for the quantity of cytokine that is bioactive. The total quantity of cytokine produced is best determined by ELISA. The ELISA kits are now commercially available for
### Table 2

**Common Bioassays Used to Quantitate Cytokine Production**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Cell Line for Bioassay (ref.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human IL-2</td>
<td>CTLL-2 (45)</td>
</tr>
<tr>
<td>Murine IL-2</td>
<td>CTLL (46)</td>
</tr>
<tr>
<td>Human IL-3</td>
<td>TF-1 cells (47)</td>
</tr>
<tr>
<td>Murine IL-3</td>
<td>NFS 60 (48)</td>
</tr>
<tr>
<td>Human IL-4</td>
<td>PHA-activated peripheral blood mononuclear cells (49)</td>
</tr>
<tr>
<td>Murine IL-4</td>
<td>CT4S or HT-2 cells (46,50)</td>
</tr>
<tr>
<td>Human IL-5</td>
<td>TF-1 cells (47)</td>
</tr>
<tr>
<td>Murine IL-5</td>
<td>Same</td>
</tr>
<tr>
<td>Human IL-6</td>
<td>T1165.85 2 1 cells (51)</td>
</tr>
<tr>
<td>Murine IL-6</td>
<td>Same</td>
</tr>
<tr>
<td>Human IL-7</td>
<td>PHA-activated peripheral blood mononuclear cells (49)</td>
</tr>
<tr>
<td>Murine IL-7</td>
<td>Same</td>
</tr>
<tr>
<td>Human GM-CSF</td>
<td>TF-1 cells (47)</td>
</tr>
<tr>
<td>Murine GM-CSF</td>
<td>NFS-60 cells (48)</td>
</tr>
<tr>
<td>Human Interferon-gamma</td>
<td>Antiviral assay (52)</td>
</tr>
<tr>
<td>Murine interferon-gamma</td>
<td>Antiviral assay (46)</td>
</tr>
<tr>
<td>Human TNF-alpha</td>
<td>Cytotoxic assay (53)</td>
</tr>
<tr>
<td>Murine TNF-alpha</td>
<td>Cytotoxic assay (53)</td>
</tr>
</tbody>
</table>

Quantitation of most murine and human cytokines (Genzyme, Boston, MA, Endogen, R&D systems). Although these kits are expensive, they usually have a sensitivity of 1–4 pg/mL, and are specific for the cytokine being tested. Bioassays are also available for many murine and human cytokines. Although they are often not as sensitive or specific as ELISA, they provide important information concerning the function of the cytokine being secreted by the tumor cells. Cell lines for bioassay of common murine and human cytokines are listed in Table 2. For all of these assays, serial dilutions are made of the tumor cell supernatants collected as described in Section 3.5. Most of the bioassays rely on cell lines that are growth factor-dependent. In these assays, the degree of proliferation of the cell lines in the presence of the serially diluted cytokines is determined by $^3$H-thymidine incorporation. A recombinant standard is also run along with the test samples to accurately quantitate the cytokine in the test samples. Because several cytokines may stimulate the same cell line, duplicate curves are often run for each sample, one curve in the presence of cytokine-blocking antibody, to evaluate the percent of proliferation that is specifically the result of that cytokine. The exceptions are: the TNF-alpha assay (which is a cytotoxic assay) and the inter-
feron-gamma assay (which is an antiviral assay). The exact procedures for performing these assays can be found in the references listed in Table 2.

24. Genes encoding cytokines are currently one of the most commonly employed genetically altered tumor vaccine strategies in preclinical models and in clinical trials. However, other gene-modified vaccine strategies, including tumor cell surface expression of MHC class I and II molecules, and costimulatory cell surface molecules (for example B7), are also under investigation. Successful gene transfer of these gene products can be assayed using cell surface staining with MAb specific for the gene product and analyzed by standard flow cytometric methods.

25. Evaluation of vector copy number should be considered, particularly in cases of suboptimal gene product expression, to determine if the problem is at the level of transcription, or owing to inadequate transduction. Vector copy number can be evaluated by Southern blot hybridization using standard procedures.

26. If the problem is owing to inadequate transduction and all of the transduction conditions have been optimized, it is possible to significantly improve on the transduction efficiency by subjecting the transduced cells to one or two more rounds of transduction.

References


Cationic Liposome-Mediated Gene Transfer to Tumor Cells In Vitro and In Vivo

Kyonghee Son, Frank Sorgi, Xiang Gao, and Leaf Huang

1. Introduction

Development of safe and effective technology for delivering functional DNA into cells in an intact organism is crucial to broad applications of gene therapy to human disease. Both viral and nonviral vectors have been developed. Of the technologies currently being studied, liposomal delivery system is particularly attractive. Cationic liposome-mediated gene transfection (lipofection), a relatively new technique pioneered by Felgner and coworkers (1), was highly efficient for transfecting cells in culture. The liposomes were composed of an equimolar mixture of a synthetic cationic lipid N-[1-(2,3,-dioleyloxy)propyl]-N,N,N,-trimethylammonium chloride (DOTMA) and a helper lipid dioleoyl-phosphatidylethanolamine (DOPE) (Fig. 1). The DOTMA/DOPE mixture (Lipofectin) forms complexes with DNA by charge interaction upon mixing at room temperature. Other cationic lipids are DOTAP, LipofectAMINE, Lipofectam, and DC-chol. The DOTAP is a diester analog of DOTMA and commercially available. LipofectAMINE and Lipofectam are polycationic lipids with a spermine head group that show increased frequency and activity of eukaryotic cell transfection (2,3). 3β-[N-(N',N'-dimethylaminoaminoethane) carbamoyl] cholesterol (DC-chol) (Fig. 1), a cationic cholesterol derivative, was introduced by Gao and Huang (4,) and is routinely used in our laboratory. The DC-chol is now commercially available but can be easily synthesized with a single-step reaction from N,N-dimethyl ethylenediamine and cholesterol chloroformate (4), and improves the efficiency of transfection with minimal toxicity. Liposomes prepared with DC-chol and DOPE (3:2 molar ratio) are stable at 4°C for at least 1 yr (unpublished data).

From: Methods in Molecular Medicine, Gene Therapy Protocols
Edited by P Robbins Humana Press Inc, Totowa, NJ
329
Complexes of plasmid DNA and DC-chol/DOPE liposomes can be easily prepared by simple mixing. A plasmid containing a reporter gene such as *Escherichia coli* chloramphenicol acetyltransferase (CAT) (5), β-galactosidase (6), or luciferase (7), under the control of a promoter of interest, is introduced into eukaryotic cells. Within a few days after transfection, the cells are harvested and the amount of reporter gene protein in the lysate is measured. This is usually done with a simple enzymatic assay. The transfection activity of the complex tested with many cell lines and primary cells depends on the cell type. Transformed epithelial cells such as epidermal carcinoma A431 cells, lung epithelial carcinoma A549 cells, embryonic kidney epithelium 293 cells, and airway epithelium IB3-1 cells are readily transfected, but fibroblast and endothelial cells are less efficiently transfected (8; unpublished data). Suspension cells including lymphoid cells are difficult to transfect using cationic liposomes, in general. Human ovarian carcinoma cells grown as subcutaneous solid tumors in SCID mice can be transfected by directly injecting plasmid DNA/liposome complex into the tumor. The level of reporter gene expression in the tumor cells was significantly elevated if the animal received a single ip injection of cisplatin 1 wk before the intratumor lipofection (9). This observation may have a profound clinical implication as it suggests a sequential, combination therapy.

Because of the apparent safety and the versatility offered by liposomal delivery system, this technology has proven important in delivering DNA for
Liposome Transfer to Tumor Cells

therapeutic purposes in humans. The DC-chol/DOPE liposomes have been used in clinical trials for cancer (10) and cystic fibrosis transmembrane conductance regulator (CFTR) (11) gene therapy.

2. Materials
1. DC-chol/ DOPE (Avanti Polar Lipids, Alabaster, AL) liposomes (2 nmol/μL).
2. Plasmids pSV2CAT (constructed by Dr. Mark Magnuson, Vanderbilt University) or pCMVCAT (constructed by Dr. Hassan Farhood, University of Pittsburgh), pCMVβgal, and pRSVLuc at 1 μg/μL in TE buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA) was amplified in *E. coli* and purified by CsCl gradient ultracentrifugation method (12).
3. Sterile tissue-culture flasks and tubes, plates, and pipets.
4. Autoclaved Pasteur pipets, microcentrifuge tubes (1.5-mL), yellow and blue tips, phosphate-buffered saline (PBS).
5. Tissue culture medium supplemented with penicillin (100 U/mL)/ streptomycin (100 mg/mL) (Gibco-BRL, Gaithersburg, MD) with or without 10% fetal bovine serum (FBS) (HyClone, Logan, UT).
6. 0.05% Trypsin plus 0.53 mM EDTA in Ca²⁺ or Mg²⁺-free sterile saline (Gibco-BRL).
7. Lysis buffer: 0.1% Triton X-100 in 0.25M Tris-HCl, pH 7.8.
8. 25-gage needle.
10. Tumor extraction buffer: 40 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 150 mM NaCl.
11. Mice of interest (animal care in accordance with the AAALAC guidelines).

3. Methods

3.1. Purification of Plasmid DNA

Growth of the bacterial culture, harvesting, and lysis of the bacteria, and purification of plasmid DNA were as described by Sambrook et al. (12).

3.2. Tissue Culture Cells and in Vitro Transfection

1. Culture cells of interest in defined medium supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO₂.
2. Trypsinize cells and plate them into 12-well plates the day before transfection such that the culture is 60–80% confluent.
3. Add appropriate quantity of cationic liposomes (10–20 nmol) to the bottom of a 1.5-mL microcentrifuge tube.
4. Add 1 mL plasmid DNA (0.5–2.0 μg) diluted with serum-free culture medium to the liposomes.
5. Incubate for 10 min at room temperature to form liposome/DNA complex.
6. Remove serum-containing medium from each well and wash the cells once with serum-free medium.
7. Add the liposome/DNA mixture to each well and incubate cells for 5 h at 37°C in 5% CO₂ (transfection).
8. Remove the serum-free medium and add 1 mL of serum-containing medium to each well.
9. Incubate the cells in 5% CO₂ for 1–2 d (posttransfection).
10. Remove serum-containing medium and wash once with PBS.
11. Add 200 μL of lysis buffer to each well and incubate 10–15 min at room temperature.
12. Collect the lysed cells in microcentrifuge tubes (ready for CAT, β-galactosidase, and luciferase assay as well as for protein assay).

3.3. Tumor Inoculation on Mice and Intratumor Transfection

1. Seed human ovarian adenocarcinoma 2008 cells (9) in 150-mm tissue culture plates and grow to confluency in complete medium.
2. Treat cells with trypsin-EDTA (Gibco-BRL), harvest, and wash with complete medium, and resuspend in sterile PBS at 2 × 10⁷ cells/mL.
3. Inject 0.1-mL aliquots SC to the flanks and back of mice using a 25-gage needle.
4. Let solid tumors grow to about 8 mm in diameter.
5. Dilute 30 μg of pUCCMVCAT in 1X TE buffer with 100 μL of 5% dextrose and mix with 30 nmol of DC-chol/DOPE liposomes (3:2, mol/mol) in 20 mM HEPES buffer, pH 7.8. Total volume equals to 145 μL.
6. Incubate the mixture for 10 min.
7. Directly inject the DNA/liposome complex into the tumor in three different sites.
8. After 48 h, sacrifice the animal and excise the tumor.
9. Homogenize the tumor in extraction buffer.
10. Lyse cells by three freeze/thaw cycles.
11. Heat the lysate at 65°C for 10 min and centrifuge at 16,000g for 10 min.
12. The supernatant is ready for protein and CAT assay, or it can be frozen at −80°C for at least 7 d.

4. Notes

Different cell lines require different optimal liposome/DNA ratio and different total amounts of complex. Defining and optimizing transfection conditions are important. In vitro transfection protocol of DC-chol/DOPE liposomes should be optimized with respect to the ratio of liposome/DNA, dose of the complex, the incubation time with cells for transfection, and posttransfection, and serum concentration (13). In vivo transfection of DC-chol/DOPE liposomes on tumor directly (9) should be optimized with respect to tumor size, number of injections on tumor, and injection volume and the ratio of lipid/complex.

1. Effects of DC-chol and DNA on in vitro transfection activity are shown in Figs. 2 and 3, respectively. Both curves are bell-shaped, indicating that it is important to define the optimal concentrations of liposomes and DNA. The transfection efficiencies are different in two figures owing to different cell density at transfection (see ref. 3). The optimal ratio of DC-chol (nmol)/DNA (μg) is between 10 and 20 for a variety of transformed epithelial cells.
**Fig. 2.** The effect of DC-chol liposomes concentration on transfection activity. The CHO cells were transfected with 1 µg pRSVLuc DNA complexed with various concentrations of DC-chol liposomes (0–20 nmol) for 7 h and cells were harvested after 37 h for luciferase assay.

**Fig. 3.** The effect of DNA concentration on transfection activity. The CHO cells were transfected with 10 nmol of DC-chol liposomes complexed with various concentrations of pRSVLuc DNA (0–2.5 µg). Transfection and posttransfection conditions were the same as Fig. 2.
2. The in vitro transfection activity increases with the amount of liposome/DNA complex added to the cells. The effect of the complex dose on transfection activity is shown in Fig. 4 for a complex of liposome/DNA ratio of 1 μg/nmol. Note that this is a suboptimal ratio that allows transfections with high complex doses and minimal toxicity.

3. Cell density (the status of the cultured cells that are seeded on plate) is another factor for a successful transfection and high level expression of foreign DNA in vitro. The efficiency reduces greatly if transfection is performed on the confluent cells (see Fig. 5).

4. The toxicity varies among cell types, and is dependent on the confluency of the cell culture. Confluent cells are more resistant to the toxicity of the liposome/DNA complex. As a compromise between high transfection activity and low toxicity, cells with 60–80% confluency are the optimum.

5. Supercoiled DNA gives higher transfection efficiency compared to nicked, covalently closed, or linear DNA.

6. pCMVCAT (an expression plasmid of CAT gene under the control of CMV early promoter) is more efficient for high level of expression than pSV2CAT (an expression plasmid of CAT gene under the control of SV40 early promoter).

7. Use serum-free medium, or very low concentration of serum if cells are not stable for at least 4–5 h in the absence of serum since the in vitro transfection activity is strongly inhibited by the presence of high concentrations of serum component. The
Fig. 5. The effect of cell density on transfection activity. A431 cells (0.1–10 × 10⁵ cells/well) were seeded in a six-well plate and incubated for 24 h. Cells were transfected for 4 h with 4 µg pUCSV2CAT and 40 nmol of DC-chol/DOPE liposomes in 2 mL of McCoy's medium without serum and then incubated for 36 h in complete medium. Cells seeded at 5–10 × 10⁵/well reached 100% confluency at the time of transfection.

DC-chol liposomes can tolerate up to 2% FBS without significantly reducing activity (4).

8. The DC-chol/DOPE liposomes and plasmid DNA should be sterile for in vitro tissue culture work.

9. Liposome/DNA complex should not be incubated for more than 15–20 min since prolonged incubation results in the formation of large complexes and reduced transfection efficiency.

10. Transfection on different-sized culture wells gives similar results when volumes were adjusted in proportion to surface area of the wells.

11. Transfectability decreases with increase in tumor size.

12. There is dose limitation of liposome/DNA complex; high dose (>complex of 300 µg DNA and 300 nmol DC-chol/DOPE liposomes) could result in tumor disruption.

13. More injection sites on a single tumor increases the transfection efficiency.

14. Best in vivo transfection is obtained when the ratio of liposome/DNA complex is 1 nmol/µg.

15. Tumors (size of 8–10 mm) have difficulty to take injection volumes of liposome/DNA complex greater than 200 µL.

16. Preinjection of cisplatin to mice 1 wk before lipofection greatly increases the transfection efficiency of solid tumors (in situ lipofection) (9). Tumor cells taken from animals injected with cisplatin are also more transfecetable in vitro as compared to cells taken from animals injected with PBS (Fig. 6).
Fig. 6. The effect of cisplatin on transfection activity of tumor cells. SCID mice bearing human ovarian 2008 tumor cells (sc solid tumor) were ip injected with cisplatin (5 mg/kg) or PBS (control). One week later, the tumors were excised and tumor cells were cultured and transfected in vitro with pUCCMVCAT DNA/DC-chol liposome complex according to the method described in the text.

Acknowledgments

This work is supported by NIH grants HL 50256, CA 59327, and DK 44935.

References


Methods for the Use of Cytokine Gene-Modified Tumor Cells in Immunotherapy of Cancer

Zhihai Qin and Thomas Blankenstein

1. Introduction

The development over the past few years has shown that gene therapy has become a true perspective. By January 1994, 63 clinical gene therapy protocols (gene therapy or marker studies) have been reviewed and most approved by U.S. control committees. Of these, 13 aim at inserting and expressing cytokine genes in tumor cells in order to use such gene-modified cells as vaccines in cancer patients. This idea is based on mouse experimental models that showed that tumor cells transfected with a variety of cytokine genes were rejected in immunocompetent animals. Tumor rejection induced by the locally produced cytokine results from an inflammatory response at the tumor site. Several observations are remarkable: the high efficacy of local cytokines to induce an antitumor response in the absence of systemic toxicity; a surprisingly large number of cytokines possess antitumor activity in this assay (IL-1, IL-2, IL-4, IL-6, IL-7, TNF, LT, IFN-γ, MCAF, G-CSF, GM-CSF, IP-10); and in several models, cytokine-producing tumors were heavily infiltrated by T-lymphocytes that contributed to tumor destruction (for review see ref. 1). The latter observation is interesting because T-cells can most specifically recognize and destroy tumor cells and are critically involved in tumor immunity. Consistently, in several tumor models, mice that were immunized by cytokine-gene transfected tumors also rejected the nontransduced parental tumor and occasionally were able to eliminate small preexisting tumor loads. Tumor immunity was dependent on T-cells. Thus, cytokine gene-modified tumor cell vaccines are thought to trigger a local immune response in cancer patients, which is directed against putative, so far unknown tumor antigens and which are effective against residual (presumably small) tumor loads (e.g., micro-
metastasis). It is important to note that these genetic approaches to cancer immunotherapy are the beginning of a developing field and still have to address some unresolved problems such as inappropriate presentation of tumor antigens by the tumor cells, tumor-induced immune suppression, tumor heterogeneity, or the question as to whether they are more effective than the previous attempts of immunotherapy. Currently, three cytokine gene therapy variations with IL-2, IL-4, TNF, IFN-γ, or GM-CSF genes are tested: transfected autologous tumor cells; transfected allogeneic tumor cells; and transfected autologous fibroblasts mixed with tumor cells as vaccine. In most cases, the cells are irradiated before injection. Results from these initial clinical trials do not exist yet.

The experimental procedure that has led to the aforementioned clinical trials is illustrated in Fig. 1. It includes rapid isolation of cytokine genes by PCR; cloning into eukaryotic expression vectors, either conventional or retroviral plasmids; gene transfer and expression of cytokine genes in tumor cells; and analysis of the tumorigenicity of the gene-modified cells in mice. Such an approach has been pursued with as many different cytokines and tumor cells as listed in Table 1 (see Note 1). In most experiments, retrovirus infection or electroporation was used for cytokine gene transfer. Here, protocols are given for detection of cytokines in the culture supernatant of transduced tumor cells, test of antitumor activity in vivo, and analysis of vaccine effects of cytokine producing tumor cells.

2. Materials

2.1. Animals

Mouse inbred strains syngeneic to most tumors used for gene transfection (e.g., BALB/c, C3H, C57BL/6, CBA, DBA/2) as well as immunodeficient BALB/c nu/nu (T-cell deficient) mice can be obtained from several breeding farms (e.g., Bomholtgard, Ry, Denmark). Usually, we use 6–8-wk-old female mice.

2.2. Cell Lines

The culture condition for the cytokine indicator cell lines is shown in Table 2. The culture media should be changed every 2–3 d. Cells grow best at concentrations between $1 \times 10^4$ and $5 \times 10^5$/mL. Suboptimal cytokine supply may lead to loss of factor-dependency.

2.3. Cell Proliferation Assay

1. D-PBS: Dulbecco’s phosphate buffered saline.
2. RPMI containing 10% FCS.
3. 11.30 Ci/mmol $^3$H Thymidine
4. 96-Well U-bottomed plates.
5. β Counter.
Fig. 1. Scheme for analysis of antitumor activity of local cytokines. Most cytokine cDNAs can easily be obtained by reverse PCR with RNA of mitogen-activated spleen cells and cloned into appropriate expression vectors. Gene transfer into tumor cells is done by either electroporation or retrovirus infection. In this chapter, protocols are given for the right lower part of the flow diagram: measuring biological activity of the transfected cytokine and analysis of local and systemic antitumor effects induced by the transfected cytokine in mice.

2.4. Cytotoxic Assay

1. RPMI/5% FCS.
2. Actinomycin D: 20 μg/mL.
3. Crystal violet solution: 0.5% crystal violet, 3% formaldehyde, 0.17% NaCl, 22.3% ethanol in water.
4. 33% Acetic acid, 1% Triton X-100.

3. Methods
3.1. Cytokine Detection

Cytokine production by gene-modified tumor cells is determined by cell lines in which proliferation is dependent on the exogenous cytokine or which are sensitive to the cytotoxic activity of a cytokine or whose phenotype changes
Table 1
Murine Tumor Cell Lines Used for Cytokine Gene Transfer (1,2)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cell line</th>
<th>Type</th>
<th>Gene transfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>CMS-5</td>
<td>Fibrosarcoma</td>
<td>hIL-2, mIFN-γ, mGM-CSF</td>
</tr>
<tr>
<td></td>
<td>C-26</td>
<td>Colon carcinoma</td>
<td>hG-CSF</td>
</tr>
<tr>
<td></td>
<td>CT-26</td>
<td>Colon carcinoma</td>
<td>mIL-2, mIFN-γ, mGM-CSF</td>
</tr>
<tr>
<td></td>
<td>J558L</td>
<td>Plasmacytoma</td>
<td>mIL-2, mIL-4, mIL-5&lt;sup&gt;a&lt;/sup&gt;,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>mIL-6&lt;sup&gt;a&lt;/sup&gt;, mIL-7,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hIL-8&lt;sup&gt;a,b&lt;/sup&gt;, mTNF,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hTNF&lt;sup&gt;b&lt;/sup&gt;, hLT&lt;sup&gt;b&lt;/sup&gt;,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>mIFN-γ, mIFN-10&lt;sup&gt;ab&lt;/sup&gt;,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>mIL-4, mIL-10</td>
</tr>
<tr>
<td></td>
<td>K485</td>
<td>Mammary adenocarcinoma</td>
<td>mIL-4, mIL-10</td>
</tr>
<tr>
<td></td>
<td>RENCA</td>
<td>Renal cell carcinoma</td>
<td>mIL-4, mGM-CSF</td>
</tr>
<tr>
<td></td>
<td>TS/A</td>
<td>Mammary adenocarcinoma</td>
<td>mIL-2, mIL-5&lt;sup&gt;a&lt;/sup&gt;, mIL-7,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>mIFN-γ, mIFN-10&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>X63</td>
<td>Plasmacytoma</td>
<td>mIL-2, mIL-4</td>
</tr>
<tr>
<td></td>
<td>MBT-2</td>
<td>Bladder carcinoma</td>
<td>hIL-2, mIFN-γ</td>
</tr>
<tr>
<td></td>
<td>FSA</td>
<td>Fibrosarcoma</td>
<td>mIL-7</td>
</tr>
<tr>
<td></td>
<td>1591-RE</td>
<td>Skin tumor, UV-induced</td>
<td>hTNF</td>
</tr>
<tr>
<td>BALB/c × C3H</td>
<td>4TO7</td>
<td>Mammary tumor</td>
<td>hIL-2</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>203-glioma</td>
<td>Glioblastoma</td>
<td>mIL-7</td>
</tr>
<tr>
<td></td>
<td>3LL</td>
<td>Lung carcinoma</td>
<td>m/hIL-2, mIL-4, hIL-6, mIFN-γ</td>
</tr>
<tr>
<td></td>
<td>B16(-F10)</td>
<td>Melanoma</td>
<td>m/hIL-2, mIL-4, mIL-5&lt;sup&gt;a&lt;/sup&gt;,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>mIL-6, mIFN-γ, hTNF, mGM-CSF, hMCAF&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>EL4</td>
<td>T-lymphoma</td>
<td>mIL-2</td>
</tr>
<tr>
<td></td>
<td>MCA-101</td>
<td>Fibrosarcoma</td>
<td>mIFN-γ&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MCA-102</td>
<td>Fibrosarcoma</td>
<td>hIL-2, hTNF&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MCA-205</td>
<td>Fibrosarcoma</td>
<td>hTNF, mIL-6</td>
</tr>
<tr>
<td></td>
<td>MCA-207</td>
<td>Fibrosarcoma</td>
<td>mIL-6</td>
</tr>
<tr>
<td></td>
<td>CBA</td>
<td>Sp1</td>
<td>mIFN-γ</td>
</tr>
<tr>
<td></td>
<td>DBA/2</td>
<td>EB</td>
<td>mTNF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ESB</td>
<td>mTNF&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P815</td>
<td>mIL-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FLC</td>
<td>mIFN-γ</td>
</tr>
<tr>
<td></td>
<td>NFS/N</td>
<td>NIH3T3</td>
<td>Oncogene transformed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/J</td>
<td>Neuroblastoma</td>
</tr>
</tbody>
</table>

<sup>a</sup>Indicates those cytokines whose expression in tumor cells did not reduce tumorigenicity, for all others antitumor activity was defined by either delayed tumor growth or tumor rejection of transfected tumor cells or by vaccine effects of modified cells.

<sup>b</sup>Unpublished observation. The letters h and m indicate human or mouse cytokine gene.
Table 2
Indicator Cells for Detection of Several Cytokines

<table>
<thead>
<tr>
<th>Detected cytokine</th>
<th>Indicator cell line</th>
<th>Culture condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>CTLL-2(^b)</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>IL-3</td>
<td>BA/F3(^b)</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>IL-4</td>
<td>CT.4S(^b)</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>IL-5</td>
<td>D13(^b)</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>IL-6</td>
<td>TEPC1033(^b)</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>IL-7</td>
<td>IXN-2B(^b)</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>FDCP-1(^b)</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>TNF</td>
<td>L929(^e)</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>LT</td>
<td>L929(^e)</td>
<td>RPMI-1640</td>
</tr>
<tr>
<td>IFN</td>
<td>WEHI13(^d)</td>
<td>RPMI-1640</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Medium</th>
<th>FCS, %</th>
<th>2-ME</th>
<th>Cytokine(^a)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>RPMI-1640</td>
<td>10</td>
<td>50 µM</td>
<td>IL-2</td>
<td>3</td>
</tr>
<tr>
<td>IL-3</td>
<td>RPMI-1640</td>
<td>10</td>
<td>No</td>
<td>IL-3</td>
<td>4</td>
</tr>
<tr>
<td>IL-4</td>
<td>RPMI-1640</td>
<td>10</td>
<td>50 µM</td>
<td>IL-4</td>
<td>5</td>
</tr>
<tr>
<td>IL-5</td>
<td>RPMI-1640</td>
<td>10</td>
<td>50 µM</td>
<td>IL-5</td>
<td>6</td>
</tr>
<tr>
<td>IL-6</td>
<td>RPMI-1640</td>
<td>10</td>
<td>50 µM</td>
<td>IL-6</td>
<td>7</td>
</tr>
<tr>
<td>IL-7</td>
<td>RPMI-1640</td>
<td>10</td>
<td>50 µM</td>
<td>IL-7</td>
<td>8</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>RPMI-1640</td>
<td>10</td>
<td>50 µM</td>
<td>IL-3</td>
<td>9</td>
</tr>
<tr>
<td>TNF</td>
<td>RPMI-1640</td>
<td>5</td>
<td>No</td>
<td>No</td>
<td>10</td>
</tr>
<tr>
<td>LT</td>
<td>RPMI-1640</td>
<td>5</td>
<td>No</td>
<td>No</td>
<td>10</td>
</tr>
<tr>
<td>IFN</td>
<td>RPMI-1640</td>
<td>10</td>
<td>No</td>
<td></td>
<td>11</td>
</tr>
</tbody>
</table>

\(^a\)50–100 U/mL (1 U is defined as the amount of cytokine required for half maximal proliferation of the indicator cells) are needed to ensure proliferation of the cells for a 3-d period at concentration of 1 x 10⁴–5 x 10⁵ cells/mL.

\(^b\)Proliferation of cells is dependent on the growth factor. [³H] incorporation is proportional to the amount of cytokine in the culture.

\(^c\)Cells are sensitive to the cytotoxic activity of the cytokine. Cell death is proportional to the amount of cytokine in the culture.

\(^d\)Cytokine induces MHC class II expression.

upon exposure to the cytokine to be tested. Alternatively, ELISAs for most cytokines are commercially available. Table 2 lists some indicator cell lines that can be used for detection of most immunotherapeutically relevant cytokines. A proliferation assay for IL-6, which is similar for other cytokines with the respective cells, and a cytotoxic assay for TNF/LT is given in Sections 3.1.1. and 3.1.2.

3.1.1. Protocol 1: Cell Proliferation Assay

1. Harvest exponentially growing TEPC1033 cells and wash three times with D-PBS.
2. Distribute 4 x 10⁴ cells/well in 100 µL RPMI/10% FCS in 96-well U-bottomed plates (Costar, Cambridge, MA). Add 100 µL/well of serial dilutions of culture supernatant to be tested or IL-6 standard in triplicates.
3. For specificity control, incubate the test supernatant with an anti-IL-6 neutralizing antibody for 30 min at 37°C (most anticytokine antibodies are sold by several suppliers and should be used according to the instructions).
4. Incubate cells for 48 h at 37°C. Then, add 0.5–1 µCi/well of a [³H]thymidine solution (NEN).
5. After 4 h incubation at 37°C, harvest the cells with a semiautomated apparatus and determine incorporated [³H]thymidine in a β counter.
3.1.2. Protocol 2: L929 Cytotoxicity Assay for TNF/LT

1. Distribute \( 3 \times 10^4 \) cells/well in 100 \( \mu L \) RPMI/5% FCS medium in 96-well, flat-bottomed plates and grow cells for 24 h.
2. Replace culture supernatant with 10 \( \mu L/well \) of a 20 \( \mu g/mL \) Actinomycin D solution and add 100 \( \mu L/well \) of dilutions of test samples, TNF standard, and controls in triplicates.
3. After 20 h incubation at 37°C, discard test supernatants and stain cells with 50 \( \mu L/well \) of crystal violet solution.
4. Wash plates with tap water thoroughly and dry for 30 min at room temperature.
5. Dissolve crystal violet bounded to surviving cells with 100 \( \mu L/well \) of a solution containing 33% acetic acid and 1% Triton X-100.
6. Measure \( OD_{595 \text{ nm}} \) absorption against blank by an ELISA reader. To confirm specificity of cytotoxicity, incubate the test supernatants first with cytokine antibodies for 30 min at 37°C, and then determine the activity.

3.2. Analysis of In Vivo Antitumor Effect of Cytokines Produced by Transduced Tumor Cells (see Note 2)

1. Harvest exponentially growing cells (see Note 3) and wash two times with D-PBS.
2. Resuspend cells in D-PBS at concentrations that 0.2 mL contain one-, five-, and 10-fold the minimal tumorigenic dose.
3. Inject tumor cells in a 0.2-mL volume subcutaneously into mice at a shaved region that allows easy monitoring of the growing tumors. The following groups should be included and each group should consist of at least five animals:
   a. Syngeneic mice injected with increasing numbers of parental cells
   b. Syngeneic mice injected with increasing numbers of mock-transfected cells.
   c. Syngeneic mice injected with increasing numbers of transduced cells producing different amounts of cytokine.
   d. Nude mice injected with parental cells and cytokine-producing cells.
   e. Syngeneic mice coinjected with a mixture of cytokine-producing and parental cells at different ratios (e.g., 1:1 and 1:10)
4. Monitor tumor size two times per week and record it as the mean value of the largest diameter and the diameter of the right angle (see Notes 4 and 5).

3.3. Analysis of Vaccine Effect of Cytokine Gene Modified Tumor Cells

This is the most critical step as it relates to gene therapy. The question is whether cytokine gene-modified tumor cells in some general form are able to serve as a vaccine that can protect mice from either a subsequent challenge with parental cells or even lead to elimination of small pre-existing tumor loads. One should keep in mind that some mouse tumor cells that have been used for vaccine experiments are inherently immunogenic before the genetic modification (12). Thus, the protocol should be designed to conclusively attribute the
vaccine effect to the transfected cytokine rather than to the immunogenicity of the tumor and to compare the strength of the vaccine effect to previous approaches of immunotherapy that have been already extensively tested in cancer patients (see Note 6).

1. Harvest exponentially growing cells (parental and cytokine-producing) and wash twice with D-PBS.
2. Resuspend cells in D-PBS in which 0.2 mL of cell suspension contain 5–10 times the minimal tumorigenic dose.
3. Inject tumor cells in a volume of 0.2 mL SC into the neck region of syngenic mice (see Note 7). Include the following groups:
   a. Leave one group of mice untreated, as control.
   b. Parental tumor cells, irradiated (5000–10,000 rad, the dose that is required for prevention of in vivo growth may depend on the tumor cells).
   c. Parental tumor cells, irradiated, admixed with 100 μg formaline-fixed Corynebacterium parvum (we have obtained it from R. North, Trudeau Institute).
   d. Parental tumor cells, viable, admixed with 100 μg formaline-fixed C. parvum. This group may be larger since we observed that part of the mice did not reject the tumor (13).
   e. Cytokine-producing cells, irradiated.
   f. Cytokine-producing cells, viable (tumor rejection can be complete, however, it is influenced by the choice of cytokine, level of cytokine secretion, and the particular tumor) (see Note 8).
4. After 2–4 wk, challenge each five mice per group with one-, five-, and 10-fold the minimal tumorigenic dose of parental tumor cells SC at the belly region. Monitor tumor growth as described in Section 3.2.

4. Notes
1. It is important to note that several cytokines transfected into different tumor cells reliably acted tumor-suppressively (e.g., IL-2, IL-4, IL-7), whereas others succeeded in some but failed in other models to suppress tumor growth (e.g., IL-6) or even stimulated tumor growth/metastasis (e.g., TNF). Furthermore, the cellular mechanisms responsible for tumor rejection can differ for the same cytokine in different tumor models (1) and different cytokines may partly activate common cellular pathways in the same tumor model (14). Tumor suppression was generally defined by host immune response and not by direct effects of the transfected cytokine on the tumor cells whose contribution, however, cannot be excluded (e.g., upregulation of MHC class I molecules by IFN-γ). Finally, antitumor activity was variously defined by partial or complete loss of tumorigenicity of transfected tumors or by the ability of transfected tumors to induce tumor immunity against challenge with the parental tumor. Often, the antitumor effect was proportional to the amount of cytokine secreted by the tumor.
2. Before gene transfer, inject tumor cells in increasing numbers into syngeneic mice (at least five per group) in order to determine the minimal dose for 100% tumor
take. A long period of in vitro culture of tumor cells may lead to change of tumorigenicity. Passaging of tumor cells in vivo may prevent such phenotypic changes.

3. Prepare in parallel parental tumor cells, mock-transfected cells (vector without cytokine gene), and several clones producing varying amounts of the transfected cytokine in order to determine the dose dependency of the antitumor effect. Make sure that the gene transfer procedure or cytokine secretion has not changed the growth rate of the cells in vitro.

4. This relatively simple readout system for antitumor activity of cytokines is possible only for subcutaneously growing tumors. Some tumor cells are highly metastatic and require additional analysis of the respective organs (15). Observation of mice over a longer period (up to 5 mo) is recommended because, under certain experimental conditions (e.g., in nude mice), tumors may grow out after a long latency period (14,16).

5. Evidence that can clearly attribute an antitumor effect to the secreted cytokine. Tumor growth of mock-transfected cells has to be similar to that of parental cells. Tumor suppression should be proportional to the level of cytokine secreted by the different clones. Tumor growth of cytokine producing cells can be restored by parallel application to mice of cytokine-neutralizing antibodies (17,18). Tumor growth of parental cells coinjected with cytokine transfectants is at least partially suppressed. Cytokine producing cells that grow as tumor after a certain latency period may have stopped cytokine production although the cells stably express the cytokine in vitro over that period (14). Tumor suppression is more obvious in syngeneic compared to immunodeficient mice. For example, cytokine-producing tumors are completely rejected in syngeneic mice; in nude mice, however, they are initially suppressed but lately grow out in most cases. This, additionally, would indicate T-cell-dependent and -independent effector mechanisms responsible for tumor suppression (14). Several investigators analyzed tumor growth in mice depleted for defined cell populations, like CD4, CD8, MAC-1, or asialo GM1 positive cells. In combination with immunohistochemical analysis of tumor tissues, this may give valid information of involved effector cells (14,17,18).

6. Tumor cell vaccines have been tested in cancer patients for quite a long time. One frequently employed approach consisted in irradiated tumor cells admixed with adjuvants Bacille Calmette-Guerin or C parvum. In animal models, such vaccines showed results comparable to those obtained by cytokine gene-modified tumor cells (19); in cancer patients it was difficult to obtain reproducible therapeutic effects (20).

7. Alternatively, vaccine cells were injected intraperitoneally. Whether the injection site influences the vaccine effect is not known.

8. The vaccine effect of viable seems to be superior to that of irradiated tumor cells (13).

Acknowledgment

This work was supported by the Deutsche Krebshilfe, Mildred-Scheel Stiftung e.V. and the BMBF.
Cytokine Gene-Modified Tumor Cells

References


Methods for Generation of Genetically Modified Fibroblasts for Immunotherapy of Cancer

Elaine M. Elder, Michael T. Lotze, and Theresa L. Whiteside

1. Introduction

Considerable evidence has accumulated indicating that cultured human or rodent tumor cells can be successfully transduced with cytokine genes and selected in the appropriate antibiotic–containing culture media. The selected transductants are generally able to secrete the cytokine coded for by the transduced gene, and in many cases, substantial levels (e.g., ng quantities) of the cytokine are produced. Using retroviral vectors, it has been possible to obtain stably transduced tumor cells with a variety of cytokine genes (1–4). These tumor cells have been used for immunotherapy of cancer in numerous animal models of tumor growth or metastasis, and more recently, in vaccination protocols in patients with cancer. One possible criticism that can be leveled at this type of vaccination approach is that cultured, genetically modified, and selected tumor cells might have phenotypic characteristics that are substantially different from those of unmodified tumor cells. Since retroviral vectors are often used for transduction, it is also possible that viral antigens expressed on transduced tumor cells contribute to the immune response generated as a result of vaccination. Also, primary cultures of human tumor cells are often difficult to establish and maintain.

To circumvent this criticism, an alternative approach to vaccination has been proposed, in which autologous fibroblasts are established and transduced with a given cytokine gene and serve as a source of the cytokine. Such transduced fibroblasts, when admixed with autologous irradiated tumor cells in a vaccine provide the cytokine needed for the effective generation of the specific immune response, one that is presumably directed at the tumor-associated antigens being presented on the tumor cells. The advantages of this proce-
dure are that autologous fibroblasts can be easily obtained and successfully cultured from nearly all patients, that autologous tumor cells need not be cultured prior to vaccination, and that gene transfer using retroviral supernatants is perfectly feasible using cultured human fibroblasts. In addition, tumor vaccines can be customized as desired by mixing a variable number of cytokine-producing fibroblasts with tumor cells, without a concern about adequate supplies of the former.

In this chapter, we describe the procedure for generation of human genetically-modified fibroblasts for clinical use in vaccination protocols. The procedure, which has been developed in our laboratory as a part of the ongoing IL-4 and IL-12 autologous fibroblast vaccination trials (5–7), is broadly applicable, allowing for gene transfer of a variety of vectors, and it is feasible for utilization in any well-established cellular laboratory.

2. Materials

All reagents and materials used in the biopsy tissue processing, culture, and harvest of fibroblasts are sterile. All culture medium supplemented with growth factors is made fresh and used within 5 wk of preparation.

1. 0.25% (w/v) Trypsin + 0.02% (w/v) ethylenediamine tetraacetic acid (EDTA) in phosphate buffered saline (PBS). Store at −20°C. Thaw just prior to use.
2. Antimicrobial wash medium (Minimal Essential Medium + 5 µg/mL fungizone + 250 µg/mL gentamicin) Store at 4°C.
4. Human AB Serum pretested for adventitious agents (HABS)
5. Fibroblast culture medium (Fibroblast Basal Medium + 10% HABS + 250 ng/mL fungizone + 50 µg/mL gentamicin + 1 ng/mL human fibroblast growth factor-Beta + 5 µg/mL insulin) Store at 4°C Use within 5 wk of supplementation.
6. 1% (w/v) Type I collagenase in MEM. Store at −20°C
7. Retroviral vector supernatant, certified for clinical use to be free of replication-competent retroviruses. Store at −70°C. Thaw immediately prior to use. Do not refreeze.
8. Protamine sulfate.
9. 0.9% (w/v) saline for injection.
10. Geneticin (G418).
11. Sterile dissecting tools (scalpels, forceps, and scissors); sterile trypsinizing flasks and Petri dishes; T25, T75, and T150 sterile, plastic culture flasks; microplate carriers with T25 flask adaptors; sterile 2-, 5-, 10-, and 25-mL pipets, and sterile 1.8-mL cryovials.

3. Method

3.1. Outgrowth of Fibroblasts from Human Skin Specimens

To establish autologous dermal fibroblasts in primary cultures, human skin is first aseptically collected at surgical resection or biopsy. Approximately 10–
20 cm\(^2\) of tissue are necessary to establish a fibroblast culture. Immediately after surgical resection, the skin is placed in antimicrobial wash medium in sterile containers. These should be prepared ahead of the resection and be available to the surgeon at the time the skin is obtained. It is necessary to label the containers with the patient's name, date of collection, and specimen type, including body site from which the skin was excised.

The tissue should be transported to the laboratory by hand, immediately after resection. A zippered specimen transport bag is used to transport the specimen, which is maintained at room temperature (24°C).

The skin is processed immediately after it arrives at the laboratory as follows:

1. If the skin was not transported in antimicrobial wash medium, transfer it to a container of antimicrobial wash medium and incubate the entire piece of skin for 30 min at room temperature.
2. Fibroblasts can be obtained by either outgrowth from small pieces of skin in explant culture or by enzymatic digestion of skin to yield a single cell suspension. To prepare skin for either explant culture or digestion, it is necessary to:
   a. Place the piece of skin in a sterile Petri dish. Cut off the outer edges of the skin with a scalpel and discard. Remove any attached fat with scissors and discard.
   b. Slice the trimmed piece of skin into small strips with scalpel and forceps, and carefully slice off the epidermis as well as connective tissue and discard. Place trimmed pieces in a separate dish. The best source of fibroblasts is the papillary region of the dermis.
   c. Mince into 2–3-mm pieces working quickly, so that the tissue does not dry out.
3. To establish explant culture, proceed as follows:
   a. Using dissecting tools, place 15–20 small pieces of trimmed skin 1–2 cm apart on the flat surface of T25 or T75 culture flasks. To improve adherence of tissue to the flask, pretreat the flat surface of the flask with HABS as follows: Add 1–2 mL of HABS to completely cover the plating surface of the flasks and incubate for 20–30 min in an atmosphere of 5% CO\(_2\) in air at 37°C. Aspirate off any residual HABS and place the pieces of tissue on the flat surface of the flask.
   b. Stand the flasks on end in the incubator for 30 min in order to allow the pieces of skin to adhere to the surface of the flask.
   c. Remove the flasks from the incubator and carefully pipet 5–10 mL of fibroblast culture medium into the bottom of the upright flasks. Do not dislodge the tissue pieces as the culture medium is added.
   d. Return the flasks to the incubator with the flat surface down. This allows the culture medium to come into contact with the tissue pieces. Culture medium must completely cover the bottom surface of the flask, but not cover the tissue.
4. For preparation of a single cell suspension by enzymatic digestion, proceed as follows:
   a. Place pieces of skin into 125-mL trypsinizing flasks with 20 mL of 1% Type I collagenase.
b Stir in a water bath at 37°C on a magnetic stirrer until the skin pieces are partially digested. Pour off the partial digest and add fresh enzyme solution as required. The digestion may be continued overnight.
c Centrifuge the cell digest and wash the cell pellet once with MEM. Count the number of cells in a hemacytometer using a trypan blue dye solution and record the total number of viable cells and the viability as the percent of unstained cells.
d. Centrifuge the cell suspension and resuspend the pelleted cells in fibroblast culture medium. Plate the cells at 0.5–1.0 x 10^6 cells/mL in T25 or T75 flasks. Incubate at 37°C in an atmosphere of 5% CO₂ in air.

3.2. Culture of Fibroblasts

Once a fibroblast culture is established from explant or single-cell digest, the culture should be maintained continuously. Cryopreservation of fibroblasts at the early stage of culture (prior to second passage) is not recommended. When the cells are in the log phase of growth, they can be cryopreserved. When there are at least 1 x 10^6 cells in the log phase of growth, transduction with a retroviral vector is performed in order to introduce the gene of choice into the fibroblasts. The vector should contain a selectable marker gene, such as the neomycin phosphotransferase gene. Successfully transduced fibroblasts are selected in culture by exposure to G418, a neomycin analog, which is toxic to cells that have not integrated the vector containing the neomycin-resistance gene along with the gene of choice. As the culture proliferates, aliquots of the cells are cryopreserved at passage two or three (i.e., in the log phase of growth).

The fibroblast cultures are handled as follows:

1. Do not disturb explants for 5–7 d, and then observe under the microscope for the outgrowth of fibroblasts. Replace medium on d 7 and continue to monitor the culture.
2. Observe cultures initiated with enzymatically digested skin microscopically on the day after plating. Change the medium and continue to monitor the cultures.
3. When outgrowth is approx 80–100% confluent in the digest-derived cultures or when outgrowth is extensive and dense around explants, trypsinize and split the cultures as follows:
   a. Decant or aspirate (by pipet) the medium from the explant cultures, dislodge the explant tissue pieces, and discard.
   b. Add the trypsin-EDTA solution to the culture flasks so that the monolayers are covered. Use the following volumes as a guide: 2–3 mL/T75 flask; 3–3.5 mL/T150 flask.
   c. Let the flasks stand at room temperature for 3 min. Tapping the side of the flask against the palm of the hand will help to detach the fibroblasts. Extend the time of incubation in the presence of trypsin if the fibroblasts are not detached. Incubation at 37°C can be used to hasten the process of the fibroblast detachment.
d. Remove the detached cells from the flask and place in a conical centrifuge tube. Rinse the flasks with MEM containing 10% HABS and add to the centrifuge tube. Serum is required to inhibit the action of trypsin.
c. Centrifuge the cell suspension at 460g for 10 min. Resuspend the cell pellet in 10 mL of fibroblast growth medium. Count viable cells and record their number and viability.
f. Adjust the concentration of cells in suspension as required for culture or cryopreservation.
g. To split the cultures, add approx 5000 cells/cm² to the culture flasks. Add a total volume of 5, 10, 20, or 30 mL of cell suspension in fibroblast growth medium to a T25, T75, T150, or T225 flask, respectively. For example, 7.5 × 10⁵ cells would be plated in a T150 flask.

4. Fibroblast cultures should be examined in an inverted tissue culture microscope every other day to monitor the rate of expansion and to detect any signs of poor growth or contamination.

3.3. Transduction and Selection of Fibroblasts

Cultured human fibroblasts in the log phase of growth are transduced with a retroviral vector containing a cytokine gene, such as IL-4, and a gene for selection, such as neomycin phosphotransferase. The successfully transduced fibroblasts are selected for resistance to neomycin by culture in the presence of G418, a neomycin analog.

1. When 2 × 10⁶ primary human fibroblasts have been established in culture and are in the log phase of growth, they are prepared for transduction. At least 1 × 10⁶ cells, representing approximately one-half of the total culture, are incubated in the presence of retroviral vector supernatant. A control (mock-transduced) culture is also established and treated exactly the same as the transduced cell culture except that medium instead of the retroviral supernatant is used.

2. To prepare fibroblast monolayers for transduction, trypsinize the cells and resuspend them in fibroblast culture medium. Plate the cells in T25 or larger flasks at 7500 cells/cm². Incubate at least 1–2 d to allow the fibroblasts to adhere to the plastic flasks and re-enter the log phase of growth.

3. When cultures are actively proliferating and are between 50 and 80% confluent, proceed with transductions as follows:

a. Aspirate the culture medium and add a volume of the retroviral vector supernatant that will give a multiplicity of infection (MOI) of at least 1. The MOI is calculated according to the following formula:

\[
\text{titer of supernatant} \times \text{volume of supernatant/number of fibroblasts}
\]

The titer of the viral supernatant is generally provided by the supplier. Add a total of 5 μg/mL protamine sulfate to the supernate used for transduction. The protamine sulfate is diluted 1:20 in 0.9% saline to prepare a working stock solution. A volume of the working stock of protamine sulfate is then added at a 1:100 dilution to the volume of viral vector supernate used for the transduc-
tion. This will produce a final 1:2000 dilution of protamine sulfate, which will give the required concentration of 5 μg/mL. For the mock transduced control culture, use an equal volume of 10% HABS in MEM with the protamine sulfate.

b. Incubate the flasks for 2–3 h at 37°C in 5% CO₂ in air. Aspirate the viral supernatant and wash 3 times with MEM. Add fresh fibroblast culture medium and continue to incubate the cultures.

4. Selection of transduced fibroblasts in 0.05–0.1 mg/mL G418 should begin no earlier than 3 d after the completion of the transduction procedure. There should be evidence of proliferation of the fibroblasts after the transduction procedure before the cells are put under selective pressure. Alternatively, selection may begin following the first passage of the transduced culture.

5. Observe the selected fibroblasts daily. Within 1 wk of exposure to the G418, there should be evidence of cell loss owing to elimination of nontransduced fibroblasts in the flasks. The G418-sensitive fibroblasts will die and become detached from the plastic surface, leaving plaques or holes in the monolayer. These dead cells will be removed when culture medium is replaced or when the cultures are passaged.

The selected cells must be monitored carefully. If there are any signs of widespread toxicity, recover the cells by removing the medium containing G418, wash the monolayers twice with fresh medium, and replace with fresh fibroblast growth medium.

Continue to culture the cells in G418. The concentration of G418 in the culture may be gradually increased depending on the degree of toxicity in the culture.

6. Transduced and transduced-selected fibroblasts are now ready to be tested for the ability to produce the relevant cytokine, e.g., IL-4. Collect culture supernatant, centrifuge to clarify, aliquot in 1-mL volumes, and store in 2-mL microvials at -70°C.

7. The ability of the fibroblasts to produce the relevant cytokine is generally determined by an ELISA assay. Using the results of the ELISA assay, calculate the units or micrograms of the cytokine being produced by 1 x 10⁶ fibroblasts in a 24-h period.

### 3.4. Harvest and Characterization of Fibroblasts for Adoptive Transfer

The preparation of gene-modified fibroblasts for use in adoptive therapy is generally adapted to the specific treatment protocol. The dose of cytokine delivered by the fibroblasts and the number of fibroblasts to be administered are the most important criteria to be considered in the harvest of the fibroblasts for adoptive transfer. The production of cytokine, for example, is measured by ELISA, and the quantity produced by the cultured cells is used to calculate the dose to be administered to the patient.

Prior to harvest and administration, several criteria for safety should be met. Safety testing is performed to document that the gene-modified fibroblasts in culture are free of replication-competent retroviruses as well as bacterial and/
or fungal contaminants. Cultures for aerobic and anaerobic bacteria, fungi, and mycoplasma are performed at least 2–3 d prior to fibroblast administration. Only the cultures that are negative for adventitious agents and free of endotoxin are accepted for adoptive transfer. S+/L- assays should be performed to document that the cells are free of helper virus.

On the day of fibroblast harvest for treatment, the cultures are tested by the Gram stain for the presence of bacteria. Fibroblasts that are free of bacteria by the Gram stain, are at least 90% viable, and produce adequate quantities of the specific gene product, are acceptable for transfer.

A general procedure for preparation of fibroblasts to be used in a vaccine protocol with autologous tumor cells includes the following steps:

1. Trypsinize the fibroblast monolayers, wash, count, and determine viability of the cells.
2. Save the supernatant from the transduced cultures and use it for the following assays
   a. Quantification of a gene product by ELISA.
   b. Safety testing for bacterial and fungal sterility, endotoxin assay, and mycoplasma testing
   c. Safety testing for detection of replication-competent retrovirus in S+/L- assays
3. Save 2–4 x 10^6 gene-modified fibroblasts for detection by PCR of the envelope gene of the helper virus and for the marker gene, i.e., neomycin phosphotransferase.
4. Irradiate the cells with a total of 5000 rads to prevent proliferation in vivo. Wash the fibroblast preparation and resuspend in 0.9% sterile saline.
5. Remove a sample of the harvested fibroblasts for a STAT Gram stain test.
6. Reserve a small number of transduced fibroblasts (both irradiated and non-irradiated) and put into culture to demonstrate:
   a. Failure to proliferate after irradiation to rule out unrestricted proliferation.
   b. Quantification of the gene product after irradiation
   The cultures are set-up in T25 flasks at 2.5 x 10^5 cells/flask in 5 mL of complete fibroblast growth medium
7. The fibroblasts may be mixed with irradiated autologous tumor for vaccine preparation. The mixture is centrifuged and the pellet is resuspended in 0.1 mL of sterile saline for intradermal injections.
8. Aspirate the resuspended fibroblasts, with or without tumor cells, into a 1-cc syringe with a 27-gage needle. Label the syringe and deliver it to the clinic site for administration to a patient.

4. Notes

1. In our experience, enzymatic digests of skin yield better fibroblast cultures than those established from explants. Suspensions obtained by digestion of skin are easier to handle and easily yield rapidly expanding fibroblasts. Digest-derived cultures can be established within 10 d compared to 21 d for explant-derived cultures.
2. The success rate for establishment of fibroblast cultures is 96% in our experience. Samples obtained from some patient samples may not generate cultures at all or may not generate cultures with a logarithmic rate of growth. These cultures are not useful for viral transduction.

3. Selection in G418 should be done with care. Concentrations of 0.05–0.1 mg/mL should be used initially for selection. Once the fibroblasts have been under selective pressure for 1 wk, the concentration of the selective agent may be gradually increased to 0.2 or 0.3 mg/mL.

4. The production of a cytokine by fibroblasts will vary depending on the percentage of transduced cells in culture that express the gene. The selection process is critical for providing a culture enriched in successfully transduced fibroblasts.

References


Methods for Gene Transfer to Synovium

Richard Kang, Paul D. Robbins, and Christopher H. Evans

1. Introduction

1.1. Rationale

Development of methods for gene transfer to synoviocytes was borne from the idea that gene therapy could be used to more effectively treat rheumatoid arthritis (RA) and other joint disorders (1). Current pharmaceutical modalities in use against RA have limited effectiveness because of problems related to inefficient targeting of drugs to the joint, as well as inefficacies of the drugs themselves. Drug delivery to the joint by traditional oral, iv, and intramuscular routes, depends on passive diffusion of the drug from the synovial vasculature into the joint space (2). Thus, high systemic concentrations of the drug are necessary to achieve therapeutic intra-articular drug levels; in chronic RA, perfusion of the synovium may be compromised (3), driving required systemic drug levels even higher. This is of major concern, as the pharmaceuticals used to treat this disease are associated with serious side effects. Further compounding these problems is the chronic nature of RA, which requires lifelong treatment with high dosages of these drugs.

As understanding of the pathophysiology of RA has unfolded, many proteins have emerged as possible antiarthritic agents (4). However, the use of such proteins is, as yet, impractical for several reasons. The biological activities of proteins do not survive oral administration. Many proteins have short half-lives in the circulation following injection, and, in any case, are not efficiently delivered to the joint for reasons alluded to above. For large molecules such as proteins, diffusion from the joint vasculature to the joint space is further compromised by the sieving effect of the synovium, through which passage falls as a function of molecular size (5,6). Although the possibility of direct intra-articular injection could overcome these problems, new difficulties...
would arise. Delivery by this method is unpleasant, not suitable for chronic administration, and the rapid clearance of proteins from the joint space (7) would require frequent administration to achieve a lasting therapeutic effect. Because of the risk of intra-articular infection, as well as the prohibitive expense of large-scale protein manufacture, intra-articular use of potentially antiarthritic proteins is not feasible.

The use of gene therapy to treat RA could overcome all of these problems (1,4,7). In this method, synoviocytes, the cells that line the joint space, are transduced with a gene (or genes) that encodes a therapeutic protein (or proteins). In so doing, the joint becomes the site of synthesis of its own drug, thus eliminating the problems associated with targeting. The highest concentration of the drug is now found in the joint space, thereby markedly reducing the possibility of side effects caused by systemic administration. By its very nature, the use of gene transfer techniques taps the great potential of proteins as antiarthritic agents, and, with stable insertion and prolonged, regulated expression of the therapeutic transgene product(s), there is the potential for a constant and long-lasting therapeutic action that would, in essence, effect a cure.

1.2. Strategies for Gene Transfer to Synovium

There are two basic strategies for transfer of genes to synovium: the direct or in vivo approach, and the indirect or ex vivo approach (1). The in vivo approach involves intra-articular injection of a vector to effect synoviocyte transduction in situ. Although this approach is more straightforward and less technically demanding, it is limited by presently available vectors. Retroviral vectors such as the Moloney murine leukemia virus derivatives are well characterized, and are by far the most commonly used vectors in human gene therapy trials (7). However, they have not been effective when injected into joints, as these vectors require cell division for infection and integration, whereas synoviocytes in situ are generally mitotically quiescent (1). Other vectors, such as adenovirus, adeno-associated virus, herpes simplex virus, and liposomes, which do not require cell division for transduction, have been limited in this approach by poor transduction efficiency, low levels of expression, transient expression, cytotoxicity, and immunogenicity (8).

The ex vivo approach involves harvest of synovium, isolation and culture of synoviocytes, transduction in vitro, and reintroduction of transduced synoviocytes into the joint via intra-articular injection. As synoviocytes divide readily in vitro, retroviral vectors are effective in transducing these cells (9). One major advantage to this approach lies in the ability to select for cells cotransduced with a selectable marker, such as neo\textsuperscript{r} (neomycin resistance), thereby allowing reintroduction into the joint of a relatively pure population of transduced cells. Using the rabbit knee model, we have developed a
method for gene transfer to synovial fibroblasts based on retroviral vectors and the ex vivo approach.

1.3. Synovium and Synovial Fibroblasts

Within the diarthrodial joint is a closed space defined by two tissues: synovium and articular cartilage. Synovium lines all the intra-articular surfaces of diarthrodial joint spaces except for those over the articulating ends of the bones that are covered by cartilage. It is a thin membrane, two-thirds of which, in normal rabbits, is covered by synoviocytes 1–3 cell layers thick overlying a relatively acellular subsynovial connective tissue that may be fibrous, adipose, or areolar depending on the anatomic location (2,10). Unlike all other tissues that line body cavities, synovium lacks a basement membrane (2,10).

Synoviocytes are a heterogeneous group of cells that consist of at least two distinct types. Type A synoviocytes resemble macrophages in their morphologic, physiologic, phagocytic, and antigenic characteristics (10,11). Type B synoviocytes are fibroblastic (2,10). The degree to which synovial fibroblasts differ from fibroblasts found elsewhere is unclear. However, human type B synoviocytes stain strongly for uridine diphosphoglucose dehydrogenase (a key enzyme in the synthesis of hyaluronan) (12) and are specifically labeled by a MAb 67 (13). The existence of other distinct cell types in the synovium is the subject of controversy, although reference to a type C synoviocyte, which is of a morphology intermediate to that of type A and type B synoviocytes, has been made (14). Of the different synovial cell types, however, only type B synoviocytes are found to grow in vitro after isolation and passage of cells from synovium (10). Thus, our ex vivo method for the retrovirus-mediated transfer of genes to synoviocytes is based on the type B synoviocyte.

1.4. Synovial Cell Transplantation

Essential to the technique of gene transfer to the synovium by the ex vivo approach is a reliable means of synovial cell transplantation. We have found intra-articular injection of synoviocytes to be an effective method (15).

As noted, the diarthrodial joint is a closed space. Thus it is expected that intra-articularly injected particulate materials, such as synoviocytes, will engage the synovium. Cartilage, the other exposed surface within the joint, is impermeable to molecules larger than 80 kDa, and cells are unable to adhere to it (10). Numerous studies have shown that particles of cartilage, dacron, carbon, gortex, metals, polymethylmethacrylate, and mineral crystals injected intra-articularly are indeed captured by the synovium (16,17). Particles smaller than approx 20 μm in diameter were phagocytosed, whereas larger particles were found lodged in the synovial interstitium. Further evidence to suggest that transplanted synoviocytes would be retained by the synovium is the expression
on synoviocytes of cell surface adhesion molecules such as integrins, CD44, and intercellular adhesion molecule-1, by which synoviocytes normally associate with their extracellular environment (2,18,19). Immunohistochemical studies using bromodeoxyuridine-labeled synoviocytes have confirmed that these intra-articularly injected cells are indeed captured by the synovium (9). Whether transplanted synoviocytes migrate out of the joint is the subject of current study in our laboratory.

1.5. Gene Transfer to Synovium: The Ex Vivo Approach

We have developed an ex vivo approach for gene transfer to the synovium using the rabbit knee model. Through this technique, we have been able to transfer the gene encoding interleukin-1 (IL-1) receptor antagonist protein (IRAP) into rabbit knee synovium and study the effects of its in vivo expression on arthropathies resulting from intra-articular injections of interleukin-1β (IL-1β) (9,20,21), a key mediator in the pathophysiology of arthritis, and on antigen-induced arthritis (22). Additionally, we are developing a model for arthritis in the rabbit knee based on transfer of a gene encoding IL-1β to the synovium.

The first step in the ex vivo approach is the harvest of synovial tissue from one knee joint of the New Zealand White rabbit. A partial synovectomy is performed on an anesthetized rabbit by sharp dissection via the medial parapatellar approach. Synoviocytes are isolated from the harvested tissue by digestion in 0.2% (w/v) clostridial collagenase. This initial isolate contains a heterogeneous population of cells including type A and type B synoviocytes, macrophages, mast cells, and red and white blood cells. Subculture of this isolate in Ham's F12 medium supplemented by 10% fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin eliminates postmitotic and nonadherent cells, leaving a homogeneous population of type B synoviocytes. These synovial fibroblasts are allowed to reach confluence and then are trypsinized and split at a 1:2 ratio. The subsequent split is at a 1:6 ratio in preparation for transduction. After this second split, cells are allowed to reach approx 60% confluency, at which time they are transduced by a retroviral vector. We have used the replication-defective retroviruses MFG and BAG, which are both derivatives of the Moloney murine leukemia virus (23–25). If the vector contains a selectable transgene, such as neo	extsuperscript{r}, which codes for neomycin phosphotransferase, transduced cells can be selected by culture in media containing G418 (a synthetic neomycin analog). This step is performed by increasing the concentration of G418 incrementally, as our experience has shown that immediate placement of these primary synoviocytes in the final concentration of G418 (0.5 mg/mL) results in excessive cell death. At concentrations higher
than 0.5 mg/mL, G418 is toxic to all of the synoviocytes regardless of successful transduction.

In preparation for autologous transplantation, the transduced, selected cells are trypsinized, washed three times in Gey’s balanced salt solution (GBSS), and approx $10^7$ cells are resuspended in 1 mL of GBSS. This cell suspension is injected intra-articularly into the rabbit knee through the patellar tendon. Experiments using radiopaque dye have confirmed the success of this technique and have established 1 mL to be a useful and safe volume for delivery of cells to the synovium (10, 15). Use of higher volumes entails risk of capsular rupture.

After the transplantation of transduced synoviocytes, synovial lavage fluid can be harvested to test for expression of secreted transgene products. Lavage is necessary because the rabbit knee joint contains insufficient synovial fluid for direct aspiration. The rabbit knee is lavaged with 1 mL of GBSS using the same approach for intra-articular injection of cell suspension. In our studies of transfer of the IRAP and IL-1β genes to the synovium, commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to analyze the lavage fluid. Lavage fluid can also be analyzed for inflammatory cell counts (hemocytometry) and differentials (Wright’s stain), glycosaminoglycan content (dimethylmethylene blue assay), and so on, by methods described elsewhere (20, 26).

Further analysis of the synovial tissue can be made after sacrificing the rabbit. Transgene expression can be checked in vitro by harvesting the synovial tissue, isolating and culturing the synoviocytes as outlined earlier in this section, and analyzing the supernatant for secreted transgene products. If marker transgenes, such as neo or lacZ, have been used for transduction, these cultured synoviocytes can be selected and/or stained, respectively. The lacZ gene codes for β-galactosidase, which allows cells that express this transgene to turn blue when stained using the X-gal technique (27). This staining technique can also be applied to synoviocytes in situ although high background staining sometimes makes results of this analysis ambiguous (9).

Presence of transgene and transgene mRNA can be assessed by isolating DNA and mRNA from synovial tissue using established methods with analysis by Southern or Northern blotting, by PCR or reverse transcriptase-PCR, or by in situ hybridization techniques.

2. Materials

1. Antibiotic: Cefazolin sodium (Ancef or Kefzol) 150 mg im.
2. Anesthetic: Pentobarital (Nembutal) 30 mg/kg iv and Isoflurane, 1 L/min inhalational.
3. Suture: Polyglycolic acid (Vicryl or Dexon) 4.0 with a cutting needle.
4. Gey's balanced salt solution (GBSS) (Gibco-BRL, Gaithersburg, MD) with 1% penicillin (stock 10,000 U/mL) and 1% streptomycin (stock 10,000 µg/mL) (P/S) (Gibco-BRL)
5. Phosphate-buffered saline (PBS) (Gibco-BRL).
6. Clostridial collagenase grade CLS (Worthington, Freehold, NJ) 0.2% in GBSS.
7. Trypsin grade TRL (Worthington) 0.2% in GBSS
8. Polybrene (Sigma, St Louis, MO), 8 mg/mL stock.
9. Glutaraldehyde (Sigma): 0.5% in PBS Make fresh each use
10. 1 mM MgCl$_2$ in PBS.
11. 5-bromo-4-chloroindolyl-β-D-galactosidase (X-gal) solution (Sigma): 750 µL X-gal (stock 40 mg/mL in dimethylformamide), 1500 µL/mL KC solution (stock 0.82 g K$_3$Fe[CN]$_6$. 1.05 g K$_4$Fe[CN]$_6$. 3H$_2$O), 30 µL MgCl$_2$ (stock 1M), 32.45 mL PBS (store at 4°C in the dark)
12. G418 (Geneticin) (Gibco-BRL).
13. Ham’s F12 (Gibco-BRL) with 10% fetal bovine serum (grade. certified, does not require heat inactivation) (Gibco-BRL) and 1% P/S (Gibco-BRL).

3. Methods

3.1. Harvest of Synovium from the New Zealand White Rabbit

1. Administer preoperative prophylactic antibiotics to the rabbit.
2. Anesthetize the rabbit.
3. Shave, prep, and drape the knee using sterile technique.
4. Make a 3-cm medial parapatellar incision through the skin extending 1 cm below the joint line.
5. Continue the incision through the outer joint capsule, centering the incision between the patellar tendon and the medial collateral ligament.
6. Dissect the outer capsule from the subsynovial inner capsule superiorly as far as possible, inferiorly to the medial meniscus, medially to the infrapatellar fat pad, and laterally to the medial collateral ligament.
7. Lift the exposed subsynovial inner capsule and the attached synovium with fine-toothed forceps and excise (see Note 1).
8. Place the harvested tissue into GBSS.
9. Close the outer capsule over the exposed joint space with a running suture.
10. Close the skin with interrupted sutures.

3.2. Isolation and Culture of Synoviocytes

1. Place tissue into the inner compartment of a two-compartment digestion chamber (see Note 2) containing 10 mL of 0.2% (w/v) clostridial collagenase with constant stirring at 37°C for 2 h.
2. Use a 10-mL syringe with a 21-gage needle to aspirate the cell suspension from the outer compartment of the digestion chamber.
3. Centrifuge the cell suspension at 600g.
4. Decant the supernatant.
5. Resuspend the cells in 4 mL of medium (see Note 3).
6. Incubate the cells in a T-25 tissue culture flask at 37°C in 5% CO₂ (see Note 4).
7. Replace media every 4 d.

3.3. Passage of Synoviocytes

Passage of the cultured synoviocytes should be performed when the cells reach confluence. The first split is 1–2. The second split is 1–6 in preparation for transduction.

1. Decant medium from the T-25 tissue culture flask.
2. Add 2.5 mL of 0.25% trypsin solution.
3. Swirl the trypsin solution over the cells 10 times.
4. Discard the trypsin solution.
5. Repeat steps 2–4.
6. Incubate the cells in the residual trypsin solution for 15 min at 37°C, monitoring the cells for detachment (see Note 5).
7. Add a volume of medium appropriate to the split ratio (e.g., if splitting cells of one T-25 flask 1–2, add 8 mL media).
8. Incubate the synoviocytes in T-25 tissue culture flasks at 37°C in 5% CO₂.
9. Replace media every 4 d.

3.4. Transduction of Synoviocytes

The synoviocytes are ready for viral transduction when they reach approx 60% confluence after the second split (see Note 6).

1. Decant medium from the flasks.
2. Add 1 mL medium containing 1 x 10⁶ viral particles and 8 μg polybrene to each flask.
3. Incubate the cells with viral particles at 37°C in 5% CO₂ for 2 h, swirling culture every 20 min.
4. Add 3 mL medium.
5. Incubate the synoviocytes in T-25 tissue culture flasks at 37°C in 5% CO₂.
6. Replace media every 4 d.

3.5. Selection and Testing of Synoviocytes for Expression of Transgene

The following three procedures can be performed on synoviocytes isolated, cultured, and transduced prior to transplantation as well as cells isolated and cultured from synovium harvested after sacrifice.

3.5.1. Neomycin Resistance Selection

This procedure should be performed starting no sooner than 48 h after transduction of synoviocytes with a vector containing transgene coding for neo⁺.
1. Replace medium with medium containing 0.25% G418.
2. Monitor daily for cell death and replace media as necessary.
3. Increase the concentration of G418 to 0.5% once cells have resumed growth (approx 2–4 d).
4. Monitor daily for cell death and replace media as necessary.
5. Repeat steps 3 and 4 once.

3.5.2. LacZ Expression

1. Aliquot 0.2 mL of cell solution into a 30-mm tissue culture grade Petri dish.
2. Add 0.8 mL medium.
3. Incubate cells at 37°C in 5% CO₂ for 48 h.
4. Rinse cells with PBS.
5. Fix cells with 0.5% glutaraldehyde at room temperature for 10 min.
6. Wash cells with 1 mM MgCl₂ at room temperature for 10 min.
7. Repeat step 6.
8. Stain cells with X-gal solution at 37°C overnight (min. 2 h).
9. Replace X-gal solution with PBS.
10. Plates may be stored at 4°C for at least 2 wk.

3.5.3. Testing for Cytokine Production

This testing may be performed 3 d after transduction of synoviocytes.

1. Harvest an aliquot of supernatant from the cell culture.
2. Perform ELISA and/or bioassay

3.6. Preparation of Synoviocytes for Transplantation

1. Trypsinize the cells once they reach confluence as outlined in Section 3.3.
2. Wash the cells 3X in GBSS.
3. Resuspend cells at 10⁷ cells/mL in GBSS.

3.7. Transplantation of Cultured Transduced Synoviocytes

In an autograft procedure, the cultured transduced synoviocytes should be reimplanted into the contralateral (unoperated) knee.

1. Hold the rabbit firmly in a supine position with the knees in approx 30° of flexion.
2. Slowly inject 10⁷ synoviocytes in 1 mL GBSS through the patellar tendon into the joint space of the knee using a 1-mL tuberculin syringe with a 26.5-gage needle (see Notes 7 and 8).

3.8. Post-Transplantation Testing

3.8.1. Joint Lavage

1. Hold rabbit firmly in supine position with knees in approx 30° of flexion.
2. Slowly inject 1 mL GBSS through the patellar tendon into the joint space of the knee using a 1 mL tuberculin syringe with a 26.5-gage needle.
3. Flex and extend the knee several times through its full range of motion.
4. Aspirate the knee using the same approach, syringe, and needle as in the injection (see Note 9).

3.8.2. Harvesting and Testing of Synovium

1. Sacrifice the rabbit
2. Reflect skin from the knee.
3. Separate the knee from the rabbit at the level of the midfemur proximally and the midtibia distally.
4. Transect the patellar tendon at its insertion into the tibial tubercle
5. Reflect the patellar tendon superiorly to expose the joint capsule.
6. Incise the capsule transversely at its inferior aspect.
7. Continue this incision longitudinally on the medial and lateral aspects of the capsule while maintaining tension superiorly on the patellar tendon to expose the synovium.
8. Sharply excise the exposed synovium.
9. Isolate and culture synoviocytes as outlined in Section 3.2.
10. Select and test as outlined in Section 3.5.
11. Additional analyses of synovium can be made. These include Southern and Northern blotting, PCR, and reverse transcriptase-PCR on DNA and mRNA isolated from synovium to check for presence of transgene and transgene mRNA, respectively. Sections of synovium can be analyzed by in situ hybridization and immunohistochemistry, as well as by more traditional histological methods

4. Notes

1. If the yield of synoviocytes from the harvested synovial tissue is poor, the surgical technique may be at fault. The synovium has a strong tendency to retract when cut. Therefore, it is important to grasp securely the inner capsule, and with it the synovium, while excising this tissue. A useful trick is to make a small (2 mm) transverse incision inferiorly, then slide one point of the forceps into the joint space so that the synovium and inner capsule are sandwiched between the points of the forceps. The tissue is then excised without releasing the tissue, thus preventing retraction of the synovium.
2. The two-compartment digestion chamber is described in detail by Green (28). The purpose of its use is to separate the cells from extracellular debris. In lieu of this technique, synovial tissue may be digested in a single chamber vessel under the same conditions as described in Section 3.2. and filtered through a nylon monofilament mesh of 45-μm pore size.
3. When resuspending cells, use only the smallest amount of medium possible initially to prevent formation of clumps of cells, which are difficult to separate once formed.
4. Given the relatively low number of synoviocytes isolated from the small synovial harvest and the relative "fragility" of these cells after isolation by collagenase digestion, it is best to leave the cells undisturbed for 48 h to allow maximal adherence to the tissue culture flask.
5. During trypsinization, synoviocytes will lose the stellate/dendritic morphology that they possess in adherence, and assume a rounded shape. The cells initially will detach in clumps of rounded cells; allow the majority of cells to separate from each other before stopping trypsinization.

6. Synoviocytes are transduced with multiple transgenes by use of retroviral vectors containing multiple transgenes or by sequential transduction by multiple retroviral vectors. In sequential transduction, the second transduction should be made following selection, when applicable, and passage after the first transduction.

7. The depth of the needle stick should not exceed 1 cm during intra-articular injection, and depression of the syringe plunger should meet with little or no resistance. Resistance to advancement of the syringe plunger indicates that the tip of the needle is not in the joint space.

8. As the synovium is a well-innervated structure, intra-articular injection can be painful, especially if done rapidly. Intra-articular injection of a 1-mL vol should take 10–15 s.

9. It often requires considerable practice to retrieve a useful volume of the injected GBSS during joint lavage. The needle should not be inserted too deeply, otherwise it will penetrate the posterior capsule and may lacerate the popliteal artery. Firm massage of the suprapatellar, infrapatellar, and lateral aspects of the knee during aspiration helps to increase the amount of fluid recovered; in general, it should be possible to recover ≥ 0.5 mL of fluid. When knees are badly inflamed, lavage is often difficult because of the presence of large numbers of leukocytes, fibrin, and other debris in the joint. Under such conditions, the only recourse is to sacrifice the animal and recover the GBSS surgically.

References


Methods for Adenovirus-Mediated Gene Transfer to Synovium In Vivo

Blake J. Roessler

1. Introduction

The synovial membrane that lines diarthrodial joints is composed of a loose layer of cells (1–4 cells in depth) that overlie the surface of articular and periarticular subsynovial tissues including connective tissue, adipose tissue, tendon, bone, and articular cartilage. Using light microscopy, the synovial lining tissue appears discontinuous and lacks a clearly defined basement membrane, although the cells appear to be retained by a reticulum. Cells present below this membrane include mast cells and endothelial cells (up to 10% of the total cell population; refs. 1,2). Ultrastructural studies of the synovium have indicated that at least two major types of cells are present within the synovial membrane. Type A cells are monocytoïd in appearance and contain abundant endoplasmic reticulum with fewer vesicles, vacuoles, and mitochondria. Type B cells are fibroblastoid in appearance and contain large granules, numerous filopodia, mitochondria, and intracellular vesicles. The percentage of Type A and B cells present within adult synovium varies between species (3–5). In addition, cells with an intermediate ultrastructural appearance and morphology may exist within adult synovium (6).

The synovial membrane is a multifunctional mesothelium that participates in multiple aspects of joint physiology and host immunity in both normal and disease states. Synoviocytes produce and secrete a variety of high-mol-wt glycosaminoglycans, principally hyaluronate, that provide joint fluid with its appropriate mechanical properties of lubrication and force distribution (7,8). Synoviocytes also can express an extensive panel of proinflammatory cytokines (9–12). It has also been established that the expression of a series of cell adhesion molecules by synoviocytes is important in the recruitment and activation
of circulating lymphocytes into the synovium in diseases such as rheumatoid arthritis (13). Synoviocytes can also function as antigen-presenting cells in the context of both MHC I- and MHC-II-type receptors (6).

Thus it can be seen that the alteration of synoviocyte biology using gene transfer has potential applications for the pathophysiological analysis, and perhaps treatment, of a wide variety of articular diseases. Similar to many other organ systems, gene transfer to synovium can be accomplished using either ex vivo or in vivo techniques. In specific applications, the use of intra-articular injection techniques to access the joint space for the direct transduction of the complex epithelium that we refer to as synovium may have potential advantages over the use of isolation of synovial cells in culture for ex vivo transduction. Therefore, we have chosen to focus our efforts on the development of specific techniques that will improve our ability to target synoviocytes for gene transfer in vivo using recombinant adenoviral vectors (14).

Because of its relatively large size, the knee joint represents a convenient model system for the analysis of gene transfer to the synovial membrane. In addition, well-described animal models of synovitis can be induced within the knee joint and provide the investigator with an in vivo model that can be defined in both time and space and be applied to the analysis of transduced synovial cell biology. Among these are models of adjuvant-induced arthritis in both rats and rabbits (15–17). The reader is referred to cited references for a more comprehensive description of these animal models. The advantages of these adjuvant models are that the severity of synovitis in a specific knee joint can be modulated, and that one knee can be used as an internal control that allows for comparative analysis of biological effect within a given animal.

Although best applied to the analysis of transduced synovium within the knee joint, the methods described herein can be applied to the study of the synovial membrane present in any joint space that can be accessed using an open-bore needle that will allow the introduction of recombinant adenoviral suspension into the joint cavity.

2. Materials

2.1. Animal Strains

Male or female New Zealand White Rabbits 2.0–2.5 kg in weight are used for these experiments. Female Lewis rats 100 g in weight are also used for these experiments.

2.2. Solutions

1. Phosphate-buffered saline (PBS) is used to dilute recombinant adenovirus immediately before intra-articular injection and is used for intra-articular lavage (12.2 g NaCl, 0.27 g KCl, 1.92 g Na$_2$HPO$_4$ · 2H$_2$O, 0.34 g KH$_2$PO$_4$, double distilled water to 2 L, autoclave prior to use.)
2. A Sephadex G50 (Pharmacia, Uppsala, Sweden) column in PBS buffer is used to desalt the recombinant adenoviral suspension after purification by CsCl gradient ultracentrifugation.

3. Providone/iodine is used to disinfect the periarticular skin surface.

4. 10% neutral-buffered formalin v/v, pH 7.0 (Richard-Allan Medical, Richland, MI) is used to fix representative samples of synovial tissue.

5. OCT (Sigma, St. Louis, MO) is used to embed representative samples of synovial tissue by quick freezing in dry ice/ethanol.

6. Cal-rite (Richard-Allan Medical) is used to decalcify representative samples of bone and cartilage.

2.3. Equipment

Twenty-seven-gage sterile disposable hypodermic needles 0.5 inch in length (Becton Dickinson, Rutherford, NJ) are used for intra-articular injections in rabbits; 30-gage multiuse needles 0.25 in. in length (Becton Dickinson) are used for intra-articular injections in rats and are autoclaved prior to use.

3. Methods

3.1. Determination of Viral Particle Number

Freshly prepared suspensions of recombinant adenoviral particles are spectrophotometrically analyzed at 260 nm in order to determine the number of viral particles/mL. Absorbance times dilution x 10^2 approximates the number of adenoviral particles/mL. Additional plaque assays must be performed on fresh aliquots of these preparations in order to verify the number of plaque forming units (PFU) per mL (18). A range of doses based on particles/mL or total number of particles/joint can be used for these experiments. Generally, we have used doses in the range of 10^9–10^12 particles/mL (4). However, the optimal dose for any particular series of experiments must be determined empirically, and is dependent on both the properties of the recombinant adenoviral genomic backbone being used and the biology of the transgene being expressed.

3.2. Intra-articular Injections

3.2.1. Rabbits

Rabbits are anesthetized using a single intramuscular dose of ketamine/xylazine (40 mg/kg ketamine, 7.5 mg/kg xylazine). The area surrounding the knees is shaved and cleaned with a providone/iodine solution. Freshly prepared recombinant adenoviral suspension is then placed into a 1-cc syringe and a 27-gage needle is attached to the syringe. The inferior margin of the patella is identified by palpation and the joint space is entered in an anterior-posterior plane. The needle is advanced until the hub contacts the overlying skin and the suspension (total volume of 0.5 cc/knee) is then slowly introduced into the joint space over a period of 30 s.
3.2.2. Rats

Rats are anesthetized using a single intramuscular dose of ketamine/xylazine. The area surrounding the knees is shaved and cleaned with a providone/iodine solution. Fresh recombinant adenoviral suspension is then placed into a 1-cc syringe and a 30-gage needle is attached to the syringe. The knee is held in 30° of flexion and the inferior margin of the patella is identified by palpation; the joint space is entered in an anterior-posterior plane. The needle is advanced until the hub contacts the overlying skin and the suspension (total volume of 0.1 cc/knee) is then slowly introduced into the joint space.

3.3. Analysis of Transduced Synovium

3.3.1. Rabbits

Rabbits are euthanized using approved techniques and the skin surrounding the knee is sharply dissected away from underlying fascia.

3.3.1.1. Intra-Articular Lavage

Using a scalpel, an incision is made along the medial aspect of the patellofemoral ligament from the tibial plateau to the superior margin of the patella. The patellofemoral ligament is reflected laterally and the joint space is exposed. The joint space is lavaged with 1–2 cc of PBS in 0.5-mL aliquots and the lavage fluid is aspirated from the joint cavity using a disposable transfer pipet. Cellular debris are removed by centrifugation and the recovered lavage fluid is frozen at −70°C until use.

3.3.1.2. Isolation of Articular Tissue and Synovium

In order to provide a method to ensure that comparable samples of synovium are recovered from each experimental animal, a section of the patellofemoral ligament is removed en bloc. After intra-articular lavage has been completed, the patellofemoral ligament is transected along its insertion onto the tibial plateau and then incised along the lateral aspect of the ligament. The entire patellofemoral ligament is then transected 1 cm above the superior margin of the patella. Using sharp dissection, this rectangular section of tissue can be subdivided into smaller samples that are frozen in O.C.T. compound embedding medium (Tissue-Tek, Miles Inc., Elkhart, NJ) or placed directly into 10% buffered formalin. Sections obtained from these tissue blocks can then be analyzed using standard histologic and immunohistologic techniques.

3.3.2. Rats

Rats are euthanized using approved techniques and the skin surrounding the knee is sharply dissected away from the underlying tissue.
3.3.2.1. ISOLATION OF ARTICULAR TISSUE AND SYNOVUM

Using sharp dissection, the entire knee joint, including periarticular muscle and fascia, is removed en bloc. A circumferential incision to the level of periosteum is made approx 1.5 cm above and below the superior and inferior margins of the knee joint. Using heavy scissors or a saw blade, the femur is transected 1 cm above the superior margin of the patella and the tibia/fibula are transected 1 cm below the tibial plateau. The entire block of tissue can then be immersed in 10% buffered formalin. Following decalcification using conventional methods, the sample can be divided into representative tissue blocks that can then be analyzed using standard histologic and immunohistologic techniques.

4. Notes

1. Suspensions of recombinant adenovirus should be kept on ice until immediately before use.
2. Transgene expression in vivo can be detected within 24 h postinfection; with the use of first generation E1A, E1B-deleted recombinant adenoviruses probably peak at 4–7 d postinfection.
3. Alternatively, samples of transduced synovium can be dissected free of underlying subsynovial tissue using needle-nosed forceps and iridectomy shears. These strips of synovial membrane can be further cut into smaller fragments, which can then be frozen or fixed as described in Section 3.3.1.2.

References


Methods for the Use of Stromal Cells for Therapeutic Gene Therapy

Joel S. Greenberger

1. Introduction

The logic behind the use of bone marrow stromal cells in gene therapy relies heavily on data that indicate the uniqueness of the bone marrow stromal cell as a differentiated cell phenotype distinct from the embryo fibroblast, adult connective tissue fibroblast, and cells derived from other sites such as the skin, or connective tissue in muscle (Table 1) (1-15). Bone marrow stromal cells comprise the slowly proliferating supportive tissue of the hematopoietic microenvironment. The anatomic localization, biology, physiology, and growth of these cells in tissue culture have been the subject of intense investigation by experimental and clinical hematologists for several years (16-28). A major controversy in basic hematology has been the question of whether bone marrow stromal cells of the hematopoietic microenvironment are transplantable. Classic bone marrow transplantation biology (30-38) has provided significant data to indicate that the hematopoietic stem cell and committed progeny are transplantable in vivo (for review, see refs. 29,39). Techniques to transplant bone marrow stem cells have gained common acceptance in both experimental and clinical hematology. In contrast, transplantation of slowly proliferating stromal cells of the hematopoietic microenvironment has only recently been demonstrated (29,33,40-51), and confirmed by others (52). The use of bone marrow stromal cells in somatic cell gene therapy has recently become a realistic option for clinical therapeutic management.

Data to support the uniqueness of the bone marrow stromal cell phenotype has come from several sources. The physiologic function of the marrow stromal cell as a regulatory supportive structure for the hematopoietic stem cell has been shown to be a unique property, distinct from cells of other tissues...
Bone marrow stromal cells have been shown to be unique with respect to production of specific cell surface and extracellular matrix molecules (4,5,7,11,12,38); their response is unique to the addition of extracellular cytokines in response patterns biologically distinct from the response of skin fibroblastic cells, macrophages, and endothelial cells derived from other sources (2,6,8,9,14,21,32,40,45); Table 1); and the bone marrow stromal cell phenotype has been shown to be uniquely altered in genetic mutant mouse strains (27,33,42). These data add further support to the notion that there is expression of specific phenotypic characteristics in bone marrow stromal cells.

The mechanism by which bone marrow stromal cells uniquely support hematopoietic stem cells, another unique property of this cell type (44–46), has been one focus of intense investigation. The response of cells of the hematopoietic microenvironment to toxic agents that are commonly administered to cancer patients, including radiation therapy and chemotherapy, have been shown to produce biologic alteration in these cells that can be related to abnormalities of the bone marrow organ function (19,20,22–25,28,30,31,34,35,39,41,43). Thus, the first central theory behind the use of bone marrow stromal cells in somatic cell gene therapy is the uniqueness of the cell phenotype. A second theoretical requirement for the use of bone marrow stromal cells in gene therapy

### Table 1
**Biologic Property Unique to Bone Marrow Stomal Cells**

<table>
<thead>
<tr>
<th>Property</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differentiation to osteoblasts in DCS or in bFGF</td>
<td>9,53</td>
</tr>
<tr>
<td>SM actin isoform detected similar to vascular smooth muscle cell</td>
<td>13</td>
</tr>
<tr>
<td>GAP junction connexins detected between cells</td>
<td>12</td>
</tr>
<tr>
<td>Stimulate proliferation of CD34++/CD38− cells</td>
<td>11</td>
</tr>
<tr>
<td>Produce and respond to PDGF</td>
<td>10</td>
</tr>
<tr>
<td>Adipocyte phenotype stimulated by corticosteroid adipocyte phenotype,</td>
<td>8,16</td>
</tr>
<tr>
<td>inhibited by IL-11</td>
<td></td>
</tr>
<tr>
<td>Release of novel factors increase proliferation induced by SCF, IL-3, LIF, G-CSF using human lin−/CD34+/HLADr cells</td>
<td>7</td>
</tr>
<tr>
<td>Interferon induces apoptosis</td>
<td>6</td>
</tr>
<tr>
<td>Produce novel 37-kDa membrane adhesive protein</td>
<td>5</td>
</tr>
<tr>
<td>Fibronectin variant detected unique to stromal cells EDa domain,</td>
<td>4</td>
</tr>
<tr>
<td>lacks EDβ domain</td>
<td></td>
</tr>
<tr>
<td>Support primary plasmacytoma growth</td>
<td>3</td>
</tr>
<tr>
<td>Human stromal cells include adventitial- reticular cells with NGF receptor</td>
<td>15</td>
</tr>
<tr>
<td>Mouse stromal cells show H513E3 antibody positivity</td>
<td>2</td>
</tr>
<tr>
<td>Barrier cell function with contractile actin microfilaments</td>
<td>1</td>
</tr>
</tbody>
</table>
Therapeutic Use of Stromal Cells

involves demonstration of the biological stability of these cells once transplanted, their capacity for expansion in vitro, and their capacity for expression of transgenes. Basic research in tissue culture expansion of bone marrow stromal cells led not only to the demonstration of transplantability of the expanded clonal lines (29,33), but also to transplantation of the noncloned, expanded fresh cultures (47–50). Lack of detectable malignant transformation of expanded stromal cells in culture following reinoculation into experimental animals was also a critical finding that led to the potential therapeutic use of this cell phenotype (47–50).

2. Materials

1. To expand bone marrow stromal cells in vitro for transgene insertion and preparation for reinfusion, several sources of tissue culture medium as well as conventional laboratory tissue culture facilities are required. Experiments have been carried out using mouse, dog, and human bone marrow. Since no clinical protocols have been initiated to date using transgene expressing human marrow stromal cells, the methods for this technique of expansion of human stromal cells rely on reference to those previously published methods for mouse cell culture (44).

2. Bone marrow stromal cell lines such as the GBL-neo' line containing the neo' marker gene (29) or those mouse cell lines obtained by fresh explant and expansion of uncloned lines (33), are grown in a 37°C, 7% CO₂, high humidity incubator using conventional tissue culture methods. Cells are grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal calf serum, and then passaged in 25-cm² flasks (Corning, Corning, NY), 100-cm² or 190-cm² flasks. Of particular note has been the observation over many years that plastic preparations from some biological supply companies do not support bone marrow stromal cells (16,17).

3. Materials for introduction of transgenes include equipment necessary to provide for niche irradiation of long-bone sites to 1000–1200 cGy, usually to one long-bone (Fig. 1). In previous experiments, we have irradiated mice to 2 Gy or 10 Gy to either one or both hind limbs, using total body irradiation and a previously published shielding technique (33). The irradiation of one bone site to one dose in a single fraction has been shown to facilitate iv homing of stromal cells to the cleared (irradiated) site (29). This has been carried out routinely in laboratory animals by a cesium 137 gamma cell 40 irradiator. In experiments with larger animals, a Co60 teletherapy machine, or linear accelerator, has been used. This requires the collaboration and participation of a radiation oncologist, as well as close supervision by an institutional therapy physicist and radiation safety program officer. Our most recent experiments have suggested that irradiation may not be necessary for stromal cell homing in all cases. However, at the present time, our central working hypothesis is that the enhanced homing and stability of iv injected stromal cells to the high-dose irradiated site is a requisite for the research procedure; thus, a program should include the availability of an ionizing irradiation source for focused irradiation of one marrow site.
Fig. 1. Schematic of the steps involved in development of bone marrow stromal cell line transplantation: Long-term bone marrow cultures set up according to published methods (16) are subdivided into a stromal adherent cell compartment and clonal lines are established. A reporter gene, in this case GPIA-1-neo<sup>+</sup> (29), is inserted, and selection is carried out by growing the cells in G418. These cells are then engrafted into an irradiated animal (in this case, a 30–33-g mouse) with a boost irradiation dose given to one hind limb. Mouse strains used in this have included GP1/b as recipients for GPI/a marked cells, and Sl<sup>E</sup>/Sl<sup>E</sup> and mi/mi mice for various experiments looking at mutations that lead to alterations of the hematopoietic micro-environment. Following transplantation of stromal cell lines, in vivo measurements have included hematocrit, WBC, and platelet count, and bones are then explanted following sacrifice of the animal for study of these cells at a later time. As shown in the diagram, long-term bone marrow cultures established from such transplanted mice can be tested for the number of colonies of neo<sup>+</sup>-positive stromal cells (29). This is a measure of transgene expression relative to the total number of adherent cells. The long-term bone marrow cultures themselves are then tested for colony-forming unit-spleen and colony-forming unit (granulocyte/erythroid/megakaryocyte/macrophage[CFU-GEMM], and colony-forming unit-culture composed of granulocyte/macrophage cells [CFU-GM]).
3. Method

3.1. Mouse Stromal Cell Transgene Experiments

Mouse bone marrow cells are explanted to tissue culture by procedures identical to the initial steps in the step-up of long-term bone marrow cultures (16). Detailed methods describing the technique and optimization of long-term bone marrow cultures for mouse and human cells have previously been published (16,18).

1. Briefly, the cells are flushed into 25-cm² Corning plastic flasks via a 25-gage needle inserted into the transverse section of the femur and tibia of excised bones from 30–33 g adult mice. The cells are initially cultured in prescreened lots of 25% horse serum (18), then switched to medium supplemented with fetal calf serum at weeks 3–4 (17). This is done to decrease lipid accumulation in the bone marrow stromal cells (this technique should be noted and emphasized). Horse serum is used initially to maximize adherence; however, the high lipid content and high corticosteroid content in those hemopoesis/supportive lots of horse serum also increase fat accumulation in stromal cells (18). The lipid accumulation causes the stromal cells to round up and detach. Thus, the available number of stromal cells for further expansion and transgene insertion is decreased if horse serum is continually used. The switch to fetal calf serum maintains the adherence of those cells that have already attached, but it minimizes fat uptake.

2. The addition of hydrocortisone, or other 17-hydroxycorticosteroid to the medium during the first four weeks of establishment of stromal cell cultures in the mouse, is critical (16). Whereas the mechanism of adipogenesis in marrow stromal cells is not yet completely elucidated, recent data suggest that one or more growth factors in the bone marrow microenvironment may contribute to the corticosteroid-dependent lipogenesis (8). Recent information also suggests that tenascin expression in stromal cells is down-regulated by glucocorticoids in several cell types, and this may contribute to the initial adherence of these cells (14).

3. Cells are expanded in the above conditions and then switched to fetal calf serum. At this point, several different commercially available tissue culture medias have been used to promote expansion of stromal cell lines including; Dulbecco’s Modified Eagle’s Medium, RPMI-1640, and McCoy’s Modified Medium (25). We have found that with most mouse strains and stocks, including those from mutant mouse strains, the Dulbecco’s Modified Eagle’s Medium is adequate (31,42). When bone marrow stromal cells are used in cocultivation experiments with hematopoietic cells, different tissue culture media may be required, including those supplemented by specific hematopoietic growth factors (46).

Transgene insertion is accomplished by a variety of techniques. We have found that retroviral vector transfer using a packaging-defective virus works extremely well (51).

4. Animals are prepared for reinoculation of the gene transduced cells that are selected by neo^ resistance in vitro, or selection for another selectable marker transgene (29), and then injected iv with a single cell suspension of the harvested cells. We have used 1 Gy or 10 Gy total body irradiation to the right hind limb in mice, depending on the sensitivity of specific strains of mice to
irradiation (33, Fig. 1). For transgene-expressing stromal cell transplant in most normal mouse strains, a dose of 10 Gy to the right hind limb is ideal.

5. Mice are irradiated and then immediately transplanted with \(5 \times 10^5\) individually suspended stromal cells of either clonal lines or freshly expanded noncloned cell explants. We have found that this cell number is ideal for adult 30–33 g mice based on the dose–response curves of cells injected (29). Depending on the growth factor transgene, or other secreted product transgenes being used (47–50), larger cell numbers may be required. A dose–response curve experiment similar to that previously published by us is recommended (29). In some experiments, we have injected a single cell suspension of transduced stromal cells 48 h after irradiation (33); however, the immediate injection on the same day as irradiation produces comparable results.

6. Measurement of stromal cell engraftment is carried out by use of the CFU-F assay, or colony-forming unit–fibroblast assay (19, Fig 2). This is carried out by flushing cells from the irradiated or nonirradiated limb at various times after transplant, then preparing serial dilutions of single cells, and then placing these cell lines into Plastic Petri dishes. Individual colonies are scored at limiting dilution at 7 and 14 d after incubation at 37°C Gy, 7% CO\(_2\). Plating efficiency may vary after irradiation; however, it is important not to explant cells from the irradiated limb before 6 mo to allow for repair of potentially lethal irradiation damage in the irradiated site (Fig. 3). Six months ensures that repair is reasonably complete (19). One might think that the engrafted cells would home to the irradiated site and still be recoverable immediately following explant. However, we have found that damage in situ of nontransduced stromal cells that are repairing sublethal damage in vivo, contributes to increased cell death in culture and actually prevents the survival of viable, nonirradiated, transduced cells that have homed to that site (19). Thus, we recommend a 6-mo delay before explant of marrow from the irradiated site. Experiments have been carried out to demonstrate that transduced cells will survive if explanted earlier; however, recovery is suboptimal (29).

### 3.2. Gene Transfer to Human Bone Marrow Stromal Cells

The methods for growing human stromal cells in tissue culture have been published by a number of laboratories. In the method we have used, bone marrow stromal cells are explanted in tissue culture in 25-cm square flasks for 3 wk with media changes every week. The medium used has been Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal calf serum and \(10^{-5}M\) hydrocortisone sodium hemisuccinate (44). To generate homogeneous stromal cell monolayers, the adherent cells from human marrow were treated differently depending on the source.

1. With human marrow, total hip or rib bone marrow fragments were obtained, and single cells were prepared from a suspension after the fragments of tissue were minced in culture with scissors and forceps. If human aspirate or needle biopsy material was obtained, the cells could be immediately trypsinized, passaged for
Measurable Parameters of Engraftment In Vitro of Hematopoietic Cells to Bone Marrow Stromal Cells

X-irradiation
Plateau Phase Non-dividing
Stromal Cells
Adherence and Proliferation "Cobblestone" Island Formation

Hematopoietic Cells

Added Hematopoietic Cells

CFU-GEMM
CFU-GM
BFUe
CFU-M

Weekly FD Nonadherent Cell Production

Tumor Formation In Vivo

F1 Cell Line Production

Fig. 2. Schematic diagram of the technique of studying bone marrow stromal cell support of hematopoiesis in vitro: Irradiated stromal cells are used as a feeder layer for assays that have been described as the long-term culture initiating cell assay, CFU-Dexter, or CFU "cobblestone" (for review, see ref. 56). Hematopoietic cells, either nonadherent cells from long-term bone marrow culture or sorted populations, are engrafted onto stromal cell cultures and cobblestone islands (36), representative of attachment sites quantitated. Nonadherent cells from these cocultures have been tested for the colony-forming assay as described in the legend to Fig. 1, or passaged in vitro to develop factor-independent cell lines (55), and then assayed for tumor formation in vivo. The final step often requires expression of an oncogene, transgene, or factor-independent phenotype induced by another experimental protocol, such as treatment with chemical carcinogen (55).
Fig. 3. Morphologic appearance of mouse long-term bone marrow cultures developed from bones removed 8 wk after transplantation of the GBl-neo' cell line to the irradiated hind limb of a C57Bl/6J (GP1/b) adult mouse using the experimental protocols described in the legend to Fig. 1: (A) Transplanted, right hind limb. (B) Control, right hind limb. (A) A culture derived from an animal transplanted with the clonal cell line. (B) An irradiated animal not given the clonal cell line. (A) Shows an active hematopoietic culture indicating stable engraftment of the stromal cells that support hemopoiesis following explant. (B) Shows residual recipient cells of macrophage or fibroblast morphology that survived the irradiation. No active hemopoiesis is seen.
Therapeutic Use of Stromal Cells

at least four passages, and then either frozen in liquid nitrogen, or used immediately for gene transfer. The morphology of these cultures varies between marrow specimens; the reason for this variation is not yet known. In prior studies, a homogenesis population of large fibroblast-like cells has been seen and is usually devoid of nonspecific esterase, or macrophage-like cells (44) Stromal cell cultures prepared by these techniques have been shown to express collagen type I, fibronectin, or adhesion molecules by indirect immunostaining techniques (44).

2. Transgene insertion is carried out by tissue culture of the cells in 2 µg/mL of dextran, or polybrene, for 24 h before the addition of a packaging-defective retrovirus containing the transgene. These techniques have previously been published in detail (51). We have found, as have others, that use of the neomycin-resistance gene and subsequent selection of the cells in G418 is greatly enhanced if the cells are cultured in medium without G418 for 2-3 d before the selection begins. We have found this is attributable to the toxicity of polybrene for the cells and the requirement of the cells to recover for several days before G418 selection begins. Electroportation and lipofection techniques have also been used for stromal cells along with other vector transfer systems that have been tested. For the stromal cell gene transfer experiments reported by us, use of retroviral vector transfer to the stromal cells is highly reproducible and, in many ways, the optimal technique (36, Fig. 4).

3.3. Injection of Gene-Transduced Stromal Cells into Mice

This technique requires tail-vein injections very similar to that for the CFU-S assay (colony cloning in its spleen) and requires iv injection of 0.1–0.2 mL single-cell suspension (for review, see ref. 33). Clumping of stromal cells can be minimized by proper trypsinization, washing, and resuspension prior to injection. Cells can be prevented from adhering by placing the syringes containing the cells in ice prior to injection. To minimize trauma to the animal, the cells should be warmed to body temperature immediately before injection.

4. Notes

1. The most common source of problems in laboratories working with bone marrow stromal cells is failure to appreciate the uniqueness of the phenotype. The cells should not be assumed to be similar to permanent embryo fibroblast cell lines such as NIH/3T3 or Balb/3T3 just because of similarities in morphology (33). Thus, respect for the uniqueness of the cell phenotype is required before initiating this type of gene therapy using bone marrow stromal cell lines.

Our laboratory has been very successful at establishing permanent stromal lines from a variety of different mouse strain marrows, including mutant mouse strains (27,42), because of the attention to detail during the first 4 wk after explant.

2. The preparation of a single-cell suspension for explant of stroma is not recommended. Although we have had success in prior experiments with single cell suspensions, the original technique of flushing clumped marrow stroma and placing it in an incubator immediately after flushing is still optimal (17). The clumped
cells initially represent several different morphologic types, including cells that look like fibroblasts, reticular adventitial cells, endothelial cells, adipocytes, and macrophages. There is significant controversy as to whether these different morphologic types represent in vivo differentiation of a common stromal cell
Therapeutic Use of Stromal Cells

progenitor that is still recognizable after explant or if they represent multiple, different cell phenotypes. Biological differences in cell phenotypes have been more important than morphologic differences (33). Therefore, it is emphasized that the cultures should be flushed, placed in the incubator, and media-changed weekly with no further manipulation of the cultures for 4 wk.

Another problem frequently encountered by investigators setting up this technique is a desire to manipulate the cultures or alter them too much during the first 4 wk.

3. Observation under an inverted microscope (not to exceed 15 min/d) is recommended, however, prolonged maintenance of cultures outside the incubator is to be avoided. The media change should be carried out by initially removing 50–75% of the tissue culture volume in a 25-cm² flask (7–8 mL); the flask should then be media-changed individually.

4. The flask should not be up-ended and left with the adherent surface exposed to air. This is very important because the cells will desiccate rapidly. Technicians are always instructed to do an initial media change one flask at a time, to replace medium by using prewarmed bottles of medium and serum in a water bath before taking them into the hood, and then pipetting the medium onto the upper surface of the flask where no cells are adherent. Flushing medium onto a surface where stromal cell colonies are growing is an excellent way to dislodge these colonies and destroy the culture. After four media changes over 4 wk, the cells are ready for passage.

5. Passaging the cells should be accomplished with careful attention to detail by using the same technique of passing one flask at a time. Initially, 1–2 passages are recommended rather than immediate serial dilution. This is different from the CFU-F assay, described previously (19), that was done to quantitate the recovery of cells rather than to establish permanent lines.

The stromal cells are passaged when they reach confluence. The stromal cells should not be permitted to pile up and overgrow culture medium. Upon reaching confluence when the cells are touching (Fig. 4B), they should be trypsinized or scrapped with a rubber policeman and passaged again. Passages, and the technique for establishing lines, are well-described in one of our most recent papers (42).

Cloning of permanent stromal cell lines for therapeutic gene transfer requires a selection of individual colonies by the technique of penicilinder, or limiting dilution cloning by Poisson statistics, both of which are identical to that previously described for embryo fibroblast lines. Once the cells have reached a stage suitable for cell line cloning (usually 12–16 wk after explant) they have adapted to tissue culture and are quite viable.

Despite their similarity to embryo fibroblasts, or other connective tissue fibroblasts, these cells have distinct phenotypic properties (previously described in Table 1). Clonal lines should be expanded and then frozen down in liquid nitrogen in 1.0 mL vol containing not less than 1 x 10⁷ cells/vial. Various techniques of liquid nitrogen freezing and thawing have been published and we have detected no significant difference in technique provided the cells are healthy at the time of freeze-down. The use of retroviral vector transfer to insert transgenes is a technique that has been previously published, and has been used successfully on stromal cell lines, as well as uncloned stromal cultures (29, 51).
References


Therapeutic Use of Stromal Cells


52. Brazolot Millan, C. L. and Carter, R. F. (1993) Apparent engraftment of transduced long-term culture adherent cells into the canine bone marrow compartment following intravenous or direct intra-marrow infusion. *Blood* 82(Suppl. 1), 428A


Suppression of the Human Carcinoma Phenotype by an Antioncogene Ribozyme

Toshiya Shitara and Kevin J. Scanlon

1. Introduction

A spectrum of oncogenes have been identified and are thought to be associated with the progression of neoplasia. These oncogenes include the following: growth factors/receptors, kinases, nuclear proteins (i.e., Fos/Jun), and tumor suppressor genes. Perturbation of one or more of these genes can transform normal cells into invasive/metastatic cancer cells. Understanding the role of these oncogenes for specific types of cancer may lead to a more rational basis for tissue-specific targets in cancer gene therapy.

Fos is one of the immediate early response genes to extracellular mitogenic stimuli (1), growth factors (2), phorbol esters (3), chemotherapy agents (4,5), and other oncogenes (6). The nuclear proteins Fos and Jun form a complex that modulates transcriptional activation of a variety of genes possessing AP-1 (activator protein 1) sites, and these genes are important in the control of cell growth (7,8). The Jun protein can form both homo- and heterodimers that bind to the AP-1 site (9,10). In contrast, Fos does not form a dimer and therefore cannot bind to DNA by itself. However, Fos can associate with Jun to generate stable heterodimers that have higher DNA-binding activity than the Jun dimers (11). AP-1 activity is elevated in neoplastic transformation along with other transforming oncogenes such as activated H-ras (12,13). The effect of H-ras on AP-1 is perceived to act through the enhanced transcriptional activity of c-jun by specific changes in the phosphorylation state of Jun (14). The inductions of c-fos and c-jun are transient; however, their effect through the signal transduction pathway can establish long-term changes in the cell growth and differentiation (15–18). Thus, targeting the fos oncogene may be a primary target for cancer gene therapy.
Several strategies have been utilized to inhibit \( c-fos \) oncogene expression: microinjection of Fos MAbs (19), \( fos \) antisense RNA (6,20,21), and \( fos \) ribozyme RNA (22,23). Antibody and antisense studies have only been able to effect partial and/or transient reversion of the \( fos \) transformed phenotype (6,19–21). In EJ (patient’s initials) human bladder carcinoma cells, the activated H-ras is thought to play an important role in tumorigenesis, invasion, and metastasis (24,25). Our previous work showed the reversal of the malignant phenotype with H-ras ribozyme in EJ cells (26,27). The \( c-fos \) ribozyme has been chosen to determine its efficacy against human tumor growth. Also, the importance of \( c-fos \) has been identified as an appropriate target for cancer gene therapy.

The designing of an effective ribozyme is complex and requires not only an understanding of nucleic acid biochemistry but also the role of the target gene in maintaining the cell phenotype. Perhaps one of the weaknesses of the early antisense studies was a limited understanding of the role of the target gene in the specific cell type. The ability to demonstrate ribozyme efficacy in vivo requires choosing the correct gene that plays a critical role in maintaining the unique cell phenotype. Two examples of this strategy were that the drug-resistant human ovarian carcinoma cells were able to maintain their phenotype by an overexpression of the \( c-fos \) oncogene (23), and that a human bladder carcinoma cell line was able to maintain an aggressive, metastatic phenotype owing to a homozygous H-ras codon 12 mutation (25–27). Down-regulation of these two oncogenes by antioncogene ribozymes produced profound phenotypic changes in the human carcinomas. However, not all genes have such a dramatic effect on maintaining unique phenotypes. Additional factors that can influence the efficacy of ribozymes are: the mRNA half-life, nuclease activity in the cell, and the stability of the protein in the cell. The cell also has the capacity to be dynamic and overcome the effects of the ribozymes by altering its cellular metabolism. The length of the ribozyme flanking sequences as well as its accessibility to the target site of the RNA are also critical for ribozyme activity. The higher the GC content of the flanking sequence, the slower the on/off rate of the ribozyme to bind to RNA and the shorter the turnover rate. Finally, the stability of the ribozyme RNA in the cell will play a role in the ribozyme efficacy.

Ribozymes can enter the cell by two methods: diffusion (endocytosis) or a vector-mediated process. If transient expression of ribozyme is required, then modifying the RNA to be more resistant to nucleases is critical, and enhancing the uptake of ribozyme can be achieved with lipofection and/or receptor-mediated carriers. If long-term expression is required, then vector-mediated uptake with a strong promoter system is critical for the stable long-term expression of ribozyme RNA. Viral vector systems have unique delivery problems and are currently limited in their usefulness in cancer gene therapy.
Ultimately, the utility of ribozymes in cancer gene therapy will be realized when we understand the following criteria: identifying critical cancer gene(s) that maintains the neoplastic phenotype; developing a tissue-specific delivery system, with a high rate of infection; and a tissue specific promoter/enhancer with long-term stable expression of the ribozyme. These problems are not technically impossible to achieve, but they must be successfully addressed before gene therapy will become a routine treatment strategy in patients.

2. Materials and Methods

1. Reagents: T4 polynucleotide kinase was obtained from New England Biolabs, Beverly, MA. Taq DNA polymerase was obtained from Perkin-Elmer/Cetus, Norwalk, CT. The (alpha \(^{32}\)P)UTP, (alpha \(^{32}\)P)dCTP, and (gamma \(^{32}\)P)ATP were obtained from New England Nuclear, Boston, MA. The RPMI-1640 media and fetal calf serum were obtained from Gibco-BRL (Gaithersburg, MD).

2. Synthetic nucleotides: Synthetic oligodeoxyribonucleotides were prepared on an Applied Biosystems (Foster City, CA) Model 380B DNA synthesizer and the sequences for the primers used in this study were previously described (23).

3. Cells: Human bladder carcinoma EJ cells were obtained from ATCC (Rockville, MD). The EJ cells were plated in triplicate at 10^4 cells/35-mm\(^2\) dish onto 0.3% agar and supplemented with 1–20% fetal bovine serum. Colonies were counted 13 d later with Giemsa dye stain. Linear regression analysis was performed on the resulting log growth curve data (6–9 d) and used to determine the slope and intercept on the log axis for cell number. Thymidine nuclear uptake studies were used to determine the rate of \((^{3}\text{H})\text{dThd}\) incorporation in acid-insoluble material. The EJ cells (2.5 × 10^3 cells/35-mm\(^2\) dish) were grown for 48 h, then incubated for 2 h with \((^{3}\text{H})\text{dThd}\) (10^6 dpm/dish), washed, acid precipitated, and counted. The EJ cell control (100%) represented 1.6 (±0.3) fmol/mg DNA/h (28).

4. Plasmid: The plasmid pMAMneo contains a 1.45-kb LTR sequence of MMTV and RSV-LTR (Clontech Lab, Palo Alto, CA). The fos ribozyme and the sequence and orientation of the ribozyme were confirmed by dideoxynucleotide sequencing of the construct as previously described (23).

5. Transfection by electroporation: Subconfluently growing EJ cells were transfected by electroporation according to a protocol provided by IBI (New Haven, CT) and previously described methods (23). Individual G418-resistant colonies were picked up, grown, and screened for expression of the fos ribozyme by the polymerase chain reaction (PCR) assay described below. The cells were challenged 8–12 wk later with G418 (1 mg/mL) to test for the presence of the neomycin gene.

6. PCR: The procedure follows that of Saiki et al. (29) as modified by Scanlon et al. (23) and a commercially available protocol (GeneAmp, Perkin-Elmer/Cetus). To detect transfected EJ with fosR, the RNA PCR assay was used as previously described (23).

7. Northern blot analysis: Messenger RNA (mRNA) isolation using the guanidium isothiocyanate and oligo(dT)-cellulose method, electrophoresis on horizontal agarose gels, hybridization, and densitometric analysis (Ambis, San Diego, CA) were performed as previously described (23,30).
Western blot analysis For immunoblotting, the MiniProtean II gel electrophoresis apparatus (Bio-Rad, Hercules, CA) and the ImmunoBlot Assay kit (Bio-Rad) were used according to the specifications of the manufacturer and a previously described method (22). The Fos MAb (Ab-1) was obtained from Oncogene Science (Manhasset, NY). α-Tubulin antibody (Oncogene Science) was used as a control.

3. Results

The criteria for successfully treating cancer cells with ribozymes require analysis by several parameters. We have used the strategy of transfecting tumor cells and using neomycin (G418) selection pressure to preselect clones for analysis. This approach does not evaluate the efficiency of transduction but it does allow for demonstration of the utility of ribozymes to target and down-regulate a specific gene expression. The criteria used to select clones for analysis are the following: morphological changes, ribozyme RNA expression (RNA-PCR assay), and Northern and Western blotting of the target gene. After this selection process, an analysis of several ribozyme-containing clones was undertaken to demonstrate the ribozyme effects on the EJ cell phenotype.

A fos ribozyme was cloned into a dexamethasone-inducible plasmid (pMAMneo) to study the transient expression of fos ribozyme RNA and its effect on EJ cells. The cells were transfected with the pMAMneo fos ribozyme plasmid by electroporation. Colonies were selected on the basis of G418-resistance and changes in the cellular morphology. Twenty of the ribozyme-expressing colonies were found to have a stable resistance to G418 when they were rechallenged with G418 (1 mg/mL).

A time-course of ribozyme RNA induction by dexamethasone was characterized (data not shown). There was some leakage of the fos ribozyme RNA in the absence of dexamethasone treatment. The fos ribozyme RNA induction increases within 2 h after dexamethasone administration, and peaks 12 h after treatment. Ribozyme RNA can be detected for 72–96 h by RNA-PCR assay. The ribozyme clones are responsive to dexamethasone for 3–9 mo. The fos ribozyme RNA expression was detected in transfected clones Rz1, Rz3, and Rz6 by the RNA-PCR assay (Fig. 1). Dexamethasone administration dramatically increased the expression of the fos ribozyme RNA in the transfected clones (Fig. 1). Expression of c-fos was correspondingly decreased by activation of the fos ribozyme (Fig. 1). Northern blot analysis indicated that the level of endogenous c-fos mRNA was decreased in ribozyme-containing clones at the 24-h time-point, whereas the phosphoglycerate kinase (PGK) mRNA remained unchanged. Western blot analysis confirmed the reduction of Fos levels while the α-tubulin levels remained unchanged (Fig. 1).
In two ribozyme clones (Rz1, Rz3), both the generation time and the rate of DNA synthesis were substantially lower than in parental EJ cells. The ribozyme clones were 26 and 29%, respectively of the control as evidenced by the (3H) thymidine incorporation assay (Table 1). The EJ cells exhibited long, spindly cells that rapidly spread to cover the growth surface, whereas the clone transfected with the ribozyme under optimal concentrations of dexamethasone were round and tended to grow in patches (Fig. 1). These effects were maintained in EJ cells for 6–8 wk and then the cells reverted back to the untreated phenotype.
Table 1  
(3H) Thymidine Incorporation Assay for EJ Ribozyme-Transfected Cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Dex, 5 µM</th>
<th>Generation time, h</th>
<th>(3H)dThd Incorporationα</th>
<th>Coloniesb 1%/20% serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>EJ</td>
<td>-</td>
<td>14.4</td>
<td>100</td>
<td>45/82</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>15.6</td>
<td>91</td>
<td>38/65</td>
</tr>
<tr>
<td>EJ Vc</td>
<td>-</td>
<td>14.4</td>
<td>78</td>
<td>42/93</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>15.4</td>
<td>84</td>
<td>32/58</td>
</tr>
<tr>
<td>EJ Rz1</td>
<td>-</td>
<td>16.8</td>
<td>67</td>
<td>28/51</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>21.6</td>
<td>26</td>
<td>3/3</td>
</tr>
<tr>
<td>EJ Rz3</td>
<td>-</td>
<td>19.2</td>
<td>73</td>
<td>34/59</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>21.6</td>
<td>29</td>
<td>1/2</td>
</tr>
<tr>
<td>EJ Rz6</td>
<td>-</td>
<td>19.2</td>
<td>—</td>
<td>31/49</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>25.2</td>
<td>—</td>
<td>2/4</td>
</tr>
</tbody>
</table>

αTo determine the rate of (3H)dThd incorporation in acid-soluble material, EJ cells (2.5 x 10^3/35 mm^2 dish) were grown for 48 h, then incubated for 2 h with (3H)dThd (10^6 dpm/dish), washed, acid precipitated, and counted as previously described (27).

bEJ cell lines (10^4 cells/35 mm^2 dish) were grown in 0.3% agar with 1% or 20% fetal bovine serum. Colonies were counted 15 d later. The control cells (EJ) equaled 82 colonies in 20% serum and 45 colonies in 1% serum.

EJ V indicates that the pMAMneo vector without fos ribozyme was transfected into EJ parent cells and used as a control.

The effects of fos ribozyme action were examined on mRNA levels and genes whose overexpression was associated with AP-1 binding sites. Genes with the signal transduction pathway for cellular transformation and cell growth were examined. Gene expression for dTMP synthase, DNA polymerase β, and topoisomerase I were decreased with that in c-fos. Gene expression for the transforming growth factor β-1 (TGFβ-1) and platelet-derived growth factor receptor (PDGFR) was also decreased (data not shown), which was associated with a decrease in cell growth characteristics as seen in Table 1.

There are several criteria that we use for vector-mediated transfection of ribozymes. As seen in Fig. 1, the effect of the ribozyme on EJ cells is reflected in morphological changes from spindly EJ cells to short, round cells in the ribozyme-containing clones. Thus it is easier to choose ribozyme expressing clones by choosing clones with the significantly altered morphology. Alternatively, a back-up system for analysis was the semiquantitative RNA-PCR assay to determine the highest level of fos ribozyme RNA (Fig. 1). However, the ultimate test for ribozyme efficacy was to determine the level of fos gene expression by Northern analysis and Fos protein expression by Western analysis. In all the ribozyme-containing clones tested, there was a direct correlation between the level of ribozyme expression and the down-regulation of the target gene by Northern blot analysis.
4. Discussion

Ribozymes are RNAs that have site-specific RNA cleavage or ligation activities that may have therapeutic applications in human diseases (31–33). The hammerhead ribozyme was derived from the plus strand of tobacco ringspot virus satellite RNA, and site-specifically cleaves RNA substrates in trans (34,35). Antioncogene hammerhead ribozymes have been designed and transfected into several human carcinoma cell lines (22,23,26,27,36–38). Because the ribozymes down-regulated only the target gene, ribozymes may be used to clarify the role of the oncogenes in the human carcinoma cells. In the previous study, we showed the efficacy of anti-H-ras ribozyme in the human bladder carcinoma EJ cells that have homozygous point mutation of H-ras codon 12 (26,27). In this study, we have demonstrated efficiency of anti-fos ribozyme and the role of fos in maintaining the EJ human carcinoma phenotype.

For long-term expression, we have used the mammalian expression vector systems such as the pHβ Apr-1 vector containing the β-actin promoter. This vector stably expressed the anti-H-ras ribozyme for 6–24 mo (26,27). The H-ras ribozymes showed continuous expression in the EJ cells, and markedly decreased tumorigenicity of EJ cells in nude mice; this means that the pHβ Apr-1 vector system is stable at least for a couple of months. In the present study, we used a dexamethasone-inducible mammalian expression vector, pMAMneo. The anti-fos ribozyme expression was induced by dexamethasone, and dramatically down-regulated the fos oncogene expression, whereas this was barely suppressed by the ribozyme without dexamethasone. The results suggest that the dexamethasone-inducible vector system is useful for the regulation of ribozyme expression (i.e., modulation of the target gene expression).

The induction of c-fos gene expression is one of the earliest nuclear responses to a wide variety of growth and differentiation factors (1–6) and suggests modulation of the cell growth through the AP-1 sites (7,8). In this study, we used the anti-fos ribozyme (fos Rz) driven by the pMAMneo vector plasmid, which limit the growth of the EJ bladder carcinoma cells.

The anti-fos ribozyme transformants (Rz1, Rz3, and Rz6) showed completely down-regulation of c-fos RNA expression with dexamethasone (Fig. 1), as well as TGF-β1 and PDGF (data not shown). Moreover, the transformants were characterized by altered cell growth, as shown by generation time, [3H]dThd incorporation, soft agar cloning assay (Table 1), and morphological changes (Fig. 1) as compared to EJ parent cells. These results suggested that the c-fos oncogene plays an important role for tumorigenicity in EJ cells. The expression of H-ras ribozyme can also inhibit growth of the EJ transformants (25–27).
In addition, the \textit{fos} ribozyme also led to down-regulation of the gene expression of TGF-\(\beta\)1 and PDGF that was associated with cell growth \((39)\). This may be explained by the fact that these two genes contain AP-1 binding sequences in their 5' regulatory sequence, and Fos/Jun complex can stimulate AP-1 site \((40,41)\).

The previous data suggest the possibility of designing site-specific hammerhead ribozymes against various substrate sequences in order to modulate target gene expression.

5. Notes

1. It appears that understanding the biology of the cells and the role of the target gene in the cell's phenotype is critical for selecting targets of ribozymes. Conversely, ribozymes can be used to determine the role of the specific gene in a metabolic pathway or cellular phenotype.
2. The ribozyme design will depend on selectivity of the target, whether the gene is overexpressed (i.e., \textit{fos}; see ref \(23\)) or mutated, such as the H-\textit{ras} codon 12 \((26)\). The effect of the ribozymes may also be rate limiting if the mutation is homozygous \((26)\) or heterozygous \((37)\) in the target gene.
3. One of the most powerful aspects of the ribozyme is its selectivity in discriminating oncogenes from proto-oncogenes \((25-28,37,38)\).
4. Ribozyme activity has been shown to be enhanced for the H-\textit{ras} ribozyme by reducing the flanking sequences from 20 to 12 bases. The shorter flanking sequences reduce specificity but enhance the turnover rate.
5. Alternatively, ribozymes can be used with conventional therapy or biological response modifiers to achieve more dramatic effects on tumors \((37)\).
6. Cellular uptake of ribozymes is limited by two problems: permeability and nucleases. Lipofection can enhance cellular uptake of ribozymes. Nuclease-resistant RNA bases or DNA bases can be incorporated into the ribozymes to enhance stability. If a transient effect on the target gene is required, this system will work well. If long-term, stable expression is required, then a vector system with a promoter/enhancer system is required.
7. Electroporation is an effective method for the introduction of DNA into the cell, but the efficiency is poor. Viral-mediated transfer of ribozymes has a higher efficacy but the tumor cells do not have a high rate of transduction. Modification of the viral surface protein \((42)\) or receptor-mediated strategies is currently being used to improve the transduction efficacy of viral vector systems for gene therapy.
8. Promoter/enhancer systems could allow for tissue-specific gene expression; however, only a few tissue-specific promoters have been identified.
9. Site-specific chromosome integration and long-term stable expression would be critical for a useful gene therapy vector.

Acknowledgments

This work was supported by the National Institutes of Health, CA 50618. We would like to thank Drs. Y. Ohta, H. Ishida, and H. Kijima for their comments on the manuscript. We would also like to thank Ms. Carol Polchow for preparing the manuscript.
Antioncogene Ribozyme Carcinoma Suppression

References

7. Curran, T., Beveren, C. V., Ling, N., and Verma, I. M. (1985) Viral and cellular pro-
talizing oncogenes activate the transcription factor PEA2. EMBO J. 7, 2475–2483.
15. Muller, R., Bravo, R., and Burekhardt, J. (1984) Induction of c-fos gene and pro-


1. Introduction

Under experimental conditions, fusion of normal and malignant cells in many different combinations most often results in the suppression of the tumorigenic phenotype of tumor cells (1). This phenomenon led to a hypothesis that the normal genome might contain "recessive cancer genes" that, when expressed, would suppress the growth of tumors (2). Since the identification and cloning of the retinoblastoma (Rb) and p53 genes, the study of what are now called tumor suppressor genes has progressed rapidly. Although tumors generally develop through multiple changes in several genes, the malignant phenotype can be reversed by the introduction of a single chromosome derived from a normal cell, suggesting that single suppressor genes may be able to overcome the effects of multiple changes related to tumor progression (3).

Among the tumor suppressor genes thus far characterized, the p53 gene is the most extensively studied (4,5). Mutations of the p53 gene and allele loss on chromosome 17p, on which this gene is located, are among the most frequent alterations identified in human malignancies. The p53 protein is highly conserved through evolution and is expressed in most normal tissues. Wild-type p53 has been shown to be involved in control of the cell cycle, transcriptional regulation, DNA replication, and induction of apoptosis. The wild-type p53 gene can suppress cell transformation and neoplastic cell growth (6–8). Recently, it was demonstrated that the growth of human lung cancer cells was suppressed by wild-type p53 that was stably expressed in the cells after retrovirus-mediated gene transfer (9).
The strategy for tumor suppressor gene therapy for cancer is to suppress the malignant phenotype of tumor cells by replacing the inactivated gene with a normal (wild-type) one to restore control of cell growth and differentiation. To effectively carry out this strategy, the therapeutic genes must be delivered efficiently and expressed at a high level in the tumor. Adenoviral vectors have many advantages over other viral vector systems and other in vitro techniques for introducing DNA into eukaryotic cells (10-12). Adenoviruses are easy to manipulate and can be obtained in high titers, $10^9-10^{12}$ plaque-forming units (PFU)/mL. They have a broad range of hosts and high rates of infectivity. Several recent animal studies have demonstrated that the use of recombinant adenoviruses for gene therapy is possible (13). Recombinant adenoviruses have been successfully administered to a variety of tissues through different routes, including intratracheal instillation (14), muscle injection (15), peripheral iv injection (16), and stereotactic inoculation to the brain (17).

We used recombinant adenovirus as a vector and wild-type p53 as a therapeutic gene to construct a p53 adenovirus and studied its efficacy as an anticancer agent in vitro and in vivo. The virus, Ad5CMV-p53, carries an expression cassette that contains human cytomegalovirus E1 promoter, human wild-type p53 cDNA, and SV40 early polyadenylation signal (18) (Fig. 1). The Ad5CMV-p53 virus mediated an efficient transfer and high-level expression of the p53 gene in human lung cancer cells (19). Growth of the lung cancer cells in which p53 was deleted or mutated was greatly inhibited by Ad5CMV-p53, whereas growth of the cell line containing wild-type p53 was less affected. Tumorigenicity tests in nude mice demonstrated that Ad5CMV-p53 prevented tumor formation. When animal tumors were treated locally with Ad5CMV-p53 and systemically with the chemotherapeutic agent cisplatin, a strong tumoricidal effect through apoptosis of tumor cells was detected (20).

The methods described in this chapter are based on our experimental designs for studying the Ad5CMV-p53 virus. These methods, as examples for research on tumor suppressor gene therapy of cancer, can be adapted and further developed for study of a specific gene in transfer and its therapeutic efficacy.

2. Materials

2.1. Determination of Gene Transfer and Expression

2.1.1. Determination of Infectivity

1. Phosphate-buffered saline (PBS): 10 mM sodium phosphate, pH 7.4, 150 mM NaCl.
2. 3.8% formalin in PBS.
3. $H_2O_2$/methanol: 1.0% $H_2O_2$ in methanol.
4. Blocking solution: 0.15% horse serum and 0.1% bovine serum albumin (BSA) in PBS.
Fig. 1. Ad5CMV-\textit{p}53 genome structure, PCR primer locations, and their sequences. The Ad5CMV-\textit{p}53 genome is about 35.4 kb divided into 100 map units (1 m.u. = 0.35 kb). The \textit{p}53 expression cassette replaced the E1 region (1.3–9.2 m.u.) of the Ad5 genome. Primer 1 is located in the first intron downstream of the human cytomegalovirus major IE gene promoter. Primer 2 is located in the SV40 early polyadenylation signal. The two primers are located 15–20 bp from either end of the \textit{p}53 cDNA and define a 1.40-kb PCR product. Primers 3 and 4 are located at 11 and 13.4 m.u., respectively, on the Ad5 genome, defining a 0.86-kb viral genome-specific PCR product.

5. Primary antibodies: Anti-\textit{p}53 monoclonal antibody (MAb) (pAb1801; Oncogene Science, Uniondale, NY) and antiactin antibody (Amersham, Arlington Heights, IL).
6. Biotinylated secondary antibody and avidin/biotinylated-peroxidase complex (ABC) reagents (Vector Laboratories, Burlingame, CA).
7. Diaminobenzidine tetrahydrochloride (DAB) stock solution: 100 mg DAB to 16.6 mL PBS (6 mg/mL final). Make 0.5-mL aliquots and store at −20°C.
8. DAB/H₂O₂ solution: Mix 0.5 mL DAB stock solution with 4.5 mL PBS with 0.1% BSA, add 100 mL of 3% H₂O₂, and let stand for 30 min. The solution must be freshly made and the tube should be wrapped with foil to protect from light.
9. Harris hematoxylin.
10. 4% Acetic acid: 8 mL acetic acid in 192 mL H₂O.
11. Saturated Li₂CO₃ solution: 6.6 g Li₂CO₃ in 500 mL H₂O.
12. 70 and 100% Ethanol solutions.
13. Xylene.
2.1.2. Detection of Intracellular Vector DNA

1. PBS: 10 mM sodium phosphate, pH 7.4, 150 mM NaCl.
2. Lysing buffer: 0.6% sodium dodecyl sulfate (SDS); 50 mM Tris-HCl, pH 7.4, 10 mM EDTA.
3. 4M NaCl solution.
4. Phenol/chloroform/isoamyl alcohol (24/24/1), pH 8.0.
5. Isopropanol.
6. 70% Ethanol.
7. 10X dNTP mix: 2 mM each of dATP, dCTP, dGTP, and dTTP in deionized H₂O.
8. 10X Polymerase chain reaction (PCR) buffer: 100 mM Tris-HCl, pH 9.0 at 25°C, 15 mM MgCl₂, 500 mM KCl, 1% Triton X-100.
9. Primers: The following two pairs of primers were designed for specific detections of the p53 insert (primers 1 and 2) and adenoviral vector (primers 3 and 4) (Fig. 1).
   a. Primer 1: 5'-GGCCCACCCCCTTGGCTTC-3'
   b. Primer 2: 5'-TTGTAACCATTATAAGCTGC-3'
   c. Primer 3: 5'-TCGTTTTCAGCAGCTGTG-3'
   d. Primer 4: 5'-CATCTCGAATCTCAAAGCGTG-3'
10. Taq DNA polymerase: 5 U/mL (Promega, Madison, WI).

2.1.3. Analysis of Gene Expression

1. SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer: 125 mM Tris-HCl, pH 6.8, 1% SDS, 1% 2-mercaptoethanol, 10% glycerol, 0.5% bromophenol blue.
2. 30% Acrylamide/0.8% bisacrylamide solution.
3. 4X Tris-HCl/SDS gel buffer: 1.5M Tris-HCl, pH 8.8, 0.4% SDS for separating gel; 1.5M Tris-HCl, pH 6.8, 0.4% SDS for stacking gel. Store solutions at 4°C.
4. Electrophoresis and transfer apparatus: mini-gel system (Bio-Rad, Hercules, CA) or equivalent.
5. Butanol for overlay in preparation of separating gel.
7. 5X SDS-PAGE running buffer: 15.1 g Tris base, 72.0 g glycine, 5.0 g SDS; add deionized H₂O to 1L. Do not adjust pH of the stock solution. The pH is 8.3 when the solution is diluted to 1X for use in the protocol.
8. Transfer buffer: 20 mM Tris/150 mM glycine, pH 8.0, 20% methanol. Store at 4°C.
10. Hybond-ECL membrane: 0.45-mm, 20 x 20 cm (Amersham).
11. 5% Biotto solution: 5% nonfat dry milk (Carnation; Nestlé, Glendale, CA) in PBS with 0.3% Tween-20.
12. Primary antibodies: Anti-p53 MAb (pAb1801; Oncogene Science) and antiactin MAb (Amersham).
15. Hyperfilm-ECL (Amersham).
16. ECL detection reagents A and B (Amersham).
2.2. Examination of the Cancer-Inhibitory Effect

2.2.1. Determination of Cell Growth

1. Trypsin-EDTA solution.
2. 0.4% Trypan blue solution. 0.4% trypan blue in 0.85% saline.
3. Hemocytometer.

2.2.2. Assay of Bystander Effect

1. Microconcentrator: Centriprep-30 and -100 (Amicon).
2. Trypsin-EDTA solution.
3. 0.4% Trypan blue solution: 0.4% trypan blue in 0.85% saline.
4. Hemocytometer.

2.3. Analyses of the Cancer-inhibitory Mechanism

2.3.1. Analysis of Cell Cycle

1. 100% Ethanol stored at -20°C
2. 0.5% Tween-20 in PBS.
3. DNase-free RNase A stock solution: 10 mg/mL in 10 mM Tris-HCl, pH 7.5, 15 mM NaCl.
4. Propidium iodide (PI) 20 mg/mL in PBS
5. “Cell Fit” program (Becton Dickinson, Rutherford, NJ).

2.3.2. Examination of Apoptosis

1. 0.01% NP-40 in PBS
2. Proteinase K solution: 20 mg/mL (Boehringer-Mannheim, Mannheim, Germany).
3. 2% H2O2 in methanol, freshly made.
4. Terminal deoxynucleotidyl transferase (TDT) buffer: 30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride
5. TDT enzyme: 17 U/mL (U.S. Biochemicals, Cleveland, OH).
7. TB buffer: 300 mM NaCl and 30 mM sodium citrate.
8. ABC reagents (Vector Laboratories).
9. Peroxidase substrate. DAB stock and working solution (as in Section 2.1.1).
10. Harris hematoxylin.
11. 4% Acetic acid: 8 mL acetic acid in 192 mL H2O.
12. Saturated Li2CO3 solution: 6.6 g Li2CO3 in 500 mL H2O.
13. 70 and 100% Ethanol solutions.
14. Xylene.
15. Cytoseal 60 (Stephens Scientific).
16. DNase I solution (for positive control): 50 mg/mL DNase I in 10 mM Tris-HCl, 140 mM sodium cacodylate, 4 mM MgCl2, and 10 mM dithiothreitol (DTT).
2.4. Evaluation of Tumor Suppressor Gene Therapy in Animal Models

2.4.1. Orthotopic Lung Cancer Model

1. Nude mice. BALB/c female nu/nu mice at age of 5 wk.
2. 100% Ethanol.
3. Disinfectant: Bacdown (Decon Laboratories, Bryn Mawr, PA) or 10% bleach.
4. Inhalation anesthetic agent: Metofane (methoxyflurane).
5. Surgical scalpel (#10) (Bard-Parker; Becton Dickinson, Franklin Lakes, NJ).
6. Forceps, hemostats, and scissors.
7. 1-cc Syringes with 25-gage needles (Bard-Parker).
8. Freshly prepared cell suspension in PBS at $1 - 5 \times 10^7$ cell/mL, stored on ice.
9. Viruses in PBS at $1 - 5 \times 10^{10}$ PFU/mL and PBS as vehicle control.
10. Stainless steel wound clip tools (Clay Adams; Becton Dickinson, Sparks, MD): Autoclip (9 mm), Autoclip Applicer, and Autoclip Remover.
12. O.C.T. compound (embedding medium for frozen tissue specimens, Miles, New Haven, CT).
13. Liquid N$_2$ and portable container.
14. 10% neutral-buffered formalin.

2.4.2. Combination Therapy Model

1. Nude mice: BALB/c female nu/nu mice at age of 5 wk.
2. 100% Ethanol.
3. Disinfectant: Bacdown (Decon Laboratories) or 10% bleach.
4. Inhalation anesthetic agent: Metofane (methoxyflurane).
5. 1-cc Syringes with 25-gage needles (Bard-Parker).
6. Freshly prepared cell suspension in PBS at $1 - 5 \times 10^7$ cell/mL, stored on ice.
7. Vernier calipers.
8. Chemotherapeutic agent: cisplatin (CDDP) at 0.5 mg/mL in PBS.
10. Liquid N$_2$ and portable container.
11. 10% Neutral buffered formalin.

3. Methods

3.1. Determination of Gene Transfer and Expression

3.1.1. Determination of Infectivity

1. Infection: To cell monolayers in 60-mm dishes at 80% confluence, add 0.5 mL viral solution at different multiplicities of infection (MOI) for 30 min at room temperature. Appropriate controls of vector and mock-infection should be applied. Add 6 mL medium and return dishes to cell incubator (37°C, 5% CO$_2$).
2. Twenty hours after infection, rinse cells with PBS and air dry.
3. Fix cells with 3.8% formalin for 5 min.
4. Treat cells with 1.0% H₂O₂ in methanol for 5 min.
5. Wash cells with PBS for 5 min.
6. Block cells with 0.15% horse serum and 0.1% BSA in PBS for 15 min.
7. Wash cells with PBS for 5 min.
8. Incubate cells with primary antibodies (1:500) in PBS with 0.1% BSA for 1 h.
9. Wash cells with PBS for 5 min with 2–3 changes.
10. Incubate cells with secondary antibody (1:1000) in PBS with 0.1% BSA for 30 min.
11. Wash cells with PBS for 5 min with 2–3 changes.
12. Incubate cells with ABC reagents at 37°C for 30 min.
13. Wash cells with PBS for 5 min with 2–3 changes.
15. Stop color reaction by washing cells with PBS for 5 min with 2–3 changes
16. Counterstain cells with Harris hematoxylin for 1 min.
17. Wash cells with deionized H₂O for 3 min with 3 changes.
18. Treat cells with 4% acetic acid for 3 min.
19. Wash cells with deionized H₂O for 3 min.
20. Treat cells with saturated Li₂CO₃ for 3 min.
21. Wash cells with deionized H₂O for 3 min.
22. Dehydrate cells with 70% ethanol for 2 min and 100% ethanol for 5 min.
23. Treat cells with xylene for 5 min.
24. Mount cells with cover slides using Cytoseal 60.

3.1.2. Detection of Intracellular Vector DNA

1. Infect cells in 60-mm dishes at appropriate MOI of virus and vector control. Use medium alone as mock-infection.
2. At different times after infection, rinse cells with PBS and quickly with deionized H₂O.
3. Add 0.75 mL lysing buffer to cell monolayers, followed by 0.25 mL 4M NaCl.
4. Mix by scraping and collect cell lysate.
5. Leave cell lysate at 4°C overnight.
6. Pellet precipitated genomic DNA at 4°C for 15 min.
7. Collect supernatant, extract with one vol of phenol/chloroform.
8. Precipitate DNA with 0.6 vol of isopropanol at –80°C for 30 min.
9. Rinse pellets with 70% ethanol, air dry pellets, and resuspend pellets with 20 mL H₂O.
10. Make multiple 31-mL PCR mixture with a ratio of 5 mL each of 10X buffer, 10X 2 mM-dNTP mix, primers (total four), and 1 mL Taq polymerase.
11. Distribute 31 mL of mixture to each PCR tube and add 19 mL DNA sample to each tube.
12. Run a 30-cycle PCR at 94°C, 0.5 min; 58°C, 1 min; and 72°C, 2 min.
13. Analyze 5 mL each of PCR products in 1.2% agarose gel (see example in Fig. 2).
Fig. 2. Agarose gel analysis of PCR products. Human lung cancer H358 cells (homozygously deleted p53) were infected with Ad5CMV-p53 and a control virus (vector only, carrying no p53 gene) at an MOI of 50 PFU/cell. Medium was used for mock infection. At 24 h postinfection, vector DNA was extracted and analyzed by PCR as described in Section 3.1.2. Five microliters of each PCR reaction were analyzed on 1.2% agarose gel. The bands from the 1.4-kb p53 insert, the 0.86-kb viral genome fragment, and the primers are indicated. Lanes 1 and 2 show the positive and negative controls, where the purified viral DNA and the DNA extracted from mock-infected cells, respectively, were used as templates. Lanes 3 and 4 show the DNA templates prepared from the cells infected with control virus and Ad5CMV-p53, respectively.

3.1.3. Analysis of Gene Expression

1. Infect cells in 60-mm dishes at appropriate MOI of virus and vector control. Use medium alone as mock-infection.
2. At different times after infection, rinse cells with PBS and quickly with deionized H2O.
3. Add SDS-PAGE sample buffer to cell monolayers at 0.2–0.5 mL/dish.
4. Collect cell lysate by scraping and boil lysate for 3 min.
5. Run 8% SDS-PAGE with 10–20 mL sample per lane in Bio-Rad mini-gel apparatus.
6. Transfer proteins from gel to Hybond-ECL membrane at 100 V for 1 h.
7. Block membrane with 5% Blotto for 20 min at room temperature or overnight at 4°C.
9. Wash membrane in Blotto for 15 min with three changes.
10. Secondary antibody reaction: Horseradish peroxidase-conjugated IgG 1:5000 in Blotto for 30 min.
Fig. 3. Western analysis of p53 expression mediated by Ad5CMV-p53. Human lung cancer H358 cells (homozygously deleted p53) were infected with Ad5CMV-p53 or a control virus (vector only, carrying no p53 gene) at an MOI of 50 PFU/cell. On postinfection days 1, 3, and 5, as indicated, cellular lysates were prepared and analyzed by Western blotting as described in Section 3.1.3. Twenty microliters of each protein sample were fractionated by SDS-PAGE. After transfer to ECL-membrane, proteins were first probed with anti-p53 and antiactin MAb in the same binding reaction, followed by probing with horseradish peroxidase-conjugated secondary antibody; they were then detected with ECL regents. An optimal exposure of developed ECL membrane is shown. Lanes 1, 3, and 5 represent samples prepared from the control-virus-infected cells. Lanes 2, 4, and 6 represent samples from cells infected with Ad5CMV-p53. Lane 7, indicated as C (control), represents the sample of mock-infected cells.

11. Wash membrane in Blotto for 10 min with three changes.
12. Add ECL detection agents (A and B) for 1 min.
14. Expose X-ray film against membrane briefly in darkroom and develop the exposed film (see example in Fig. 3).

3.2. Examination of the Cancer-Inhibitory Effect

3.2.1. Determination of Cell Growth
1. Inoculate cells at 10^5 cells/60-mm dish and incubate overnight.
2. On d 0, infect cells at 10–50 MOI of virus and vector control, with triplicate dishes for each treatment, including medium alone as mock-infection.
3. From d 1–6, count cells daily (triplicate dishes for each time point).
4. Cell counts: Rinse cells with PBS, harvest cells with trypsin-EDTA, resuspend cells in 1–5 mL medium, dilute cell suspension with 0.4% trypan blue in 0.85% saline, and count cells in hemocytometer.
5. Average cell counts for each time point and plot cell growth curves.

3.2.2. Assays of Bystander Effect

Two types of methods can be used for bystander-effect assays: cell mix and cell-free medium assays.
3.2 2.1. CELL MIX ASSAY (BATCH A)

1. Inoculate multiple dishes of cells at $10^6$ cells/60-mm dish and incubate overnight.
2. Divide cell dishes into two batches. Infect one batch of cells at 10–50 MOI of virus and vector control. Use medium alone as mock-infection. Leave the other batch untreated.
3. Twenty hours after infection, wash cells with PBS.
4. Harvest both batches of cells separately with trypsin-EDTA and suspend cells in medium.
5. Count cells in hemocytometer.
6. Mix treated cells with equal number of untreated cells and inoculate $2 \times 10^5$ cells/dish with triplicate dishes for each treatment.
7. Count cells daily for 6 d (triplicate dishes for each time point).
8. Average cell counts for each time point and plot cell growth curves.

3.2.2. CELL-FREE MEDIUM ASSAY (BATCH B)

1. Inoculate batch A cells at $2 \times 10^7$ cells/100-mm dish and incubate overnight.
2. On d 0, infect cells at 10–50 MOI of virus and vector control with one dish for each treatment, including medium alone as mock-infection.
3. On d 1, wash treated cells with PBS twice and culture the cells with new medium (12 mL/dish).
4. On d 2, inoculate batch B cells at $1 \times 10^5$ cells/60-mm dish and incubate overnight.
5. On d 3, collect culture media from each treatment of the batch A cells.
6. Centrifuge the culture media by 10,000g at 4°C for 15 min.
7. Collect supernatants (about 11 mL from each dish).
8. Fractionate each of the supernatants with Centriprep-100.
9. Dilute the concentrated supernatants (about 2 mL/fraction) with fresh medium up to 18 mL, designate as fraction $>$100 kDa.
10. Further fractionate the through-fractions with Centriprep-30.
11. Dilute the concentrated fractions (about 2 mL/fraction) and the through-fractions with fresh medium up to 18 mL, designate as fractions 30–100 kDa and <30 kDa, respectively.
12. Wash batch B cells with PBS.
13. Treat batch B cells with the fractions: 6 mL of each fraction for each of triplicate dishes of cells.
14. From d 4–9, count cells daily (triplicate dishes for each time point).
15. Average cell counts for each time point and plot cell growth curves.

3.3. Analyses of the Cancer-Inhibitory Mechanism

3.3.1. Analysis of Cell Cycle

1. Inoculate cells at $2 \times 10^6$ cells/60-mm dish and incubate overnight.
2. Infect cells at appropriate MOI of virus and vector control for different time points. Use medium alone as mock-infection.
3. Harvest cells with trypsinization and wash cells with PBS.
4. Resuspend cell pellet in 300 mL PBS.
5. Fix cells with 700 mL prechilled 100% ethanol at -20°C for 20 min.
6. Pelletize cells and wash with PBS.
7. Treat cells with 0.5% Tween-20 in PBS.
8. Wash cells with PBS.
9. Incubate cells with DNase-free RNase A (1 mg/mL) in PBS at 37°C for 15 min.
10. Pelletize cells and wash with PBS.
11. Resuspend cell pellets with 500 mL of PI/PBS solution.
12. Run cell samples in flow cytometer using "Cell Fit" program and analyze data with SOBR mathematical model (21).

3.3.2. Examination of Apoptosis

1. Infect cells in 60-mm dishes at appropriate MOI of virus and vector control. Use medium alone as mock-infection.
2. At different times after infection, rinse cells with PBS.
3. Fix cells with 4% paraformaldehyde in PBS for 5 min.
4. Incubate cells with 0.01% NP-40/PBS for 5 min.
5. Treat cells with 20 mg proteinase K/mL in PBS for 15 min.
6. Wash cells with PBS for 3 min with three changes.
7. Treat cells with 2% H$_2$O$_2$/methanol for 5 min.
8. Wash cells with PBS for 3 min with three changes.
9. Wash cells with TDT buffer for 2 min.
10. Incubate cells with TDT enzyme (1:400 dilution of 17 U/mL) and biotin-dUTP (1:100 dilution of 50 nmol) in TDT buffer at 37°C for 45 min.
11. Treat cells with TB buffer for 5 min with two changes.
12. Wash cells deionized H$_2$O for 1 min.
13. Block cells with 2% BSA.
14. Rinse cells with PBS.
15. Incubate cells with ABC reagents at 37°C for 30 min.
16. Wash cells with PBS for 5 min with 2–3 changes.
17. Incubate cells with DAB/H$_2$O$_2$ solution at 37°C for 20–30 min. Monitor development of reddish brown color under microscope.
18. Stop color reaction by washing cells with PBS for 5 min with 2–3 changes.
19. Counterstain cells with Harris hematoxylin for 1 min.
20. Wash cells deionized H$_2$O for 3 min.
21. Treat cells with 4% acetic acid for 3 min.
22. Wash cells deionized H$_2$O for 3 min.
23. Treat cells with saturated Li$_2$CO$_3$ for 3 min.
24. Wash cells deionized H$_2$O for 3 min.
25. Dehydrate cells with 70% ethanol for 2 min and 100% ethanol for 5 min.
26. Treat cells with xylene for 5 min.
27. Mount cells with cover slides using Cytoseal 60.
3.4. Evaluation of Tumor Suppressor Gene Therapy in Animal Models

3.4.1. Orthotopic Lung Cancer Model

1. Harvest tumor cells at exponential growth phase and suspend cells in PBS at a concentration of $1 \times 10^7$ cells/mL. Store on ice.
2. Irradiate nude mice at 350 cGy of $^{137}$Cs.
3. Anesthetize mice with inhalation of methoxyflurane.
4. Position mice on operating board.
5. Sterilize anterior neck area with 100% ethanol.
6. Open skin with scalpel and carefully separate muscle layers with hemostat to expose trachea.
7. Advance needle (25-gage) through tracheal wall toward bronchi and inject 0.1 mL of cells slowly.
8. Apply Autoclips to close surgical wound.
9. Return mice to cage.
10. On the third and fourth days, repeat the intratracheal injections (see step 7) with 0.1 mL/mouse of viral solutions ($1 \times 10^{10}$ PFU/mL) or equal volume of vehicle only (PBS).
11. Observe general condition of the treated mice weekly for 6–8 wk.
13. Dissect mice to harvest mediastinal block (including lungs, heart, bronchi, and trachea).
14. Grossly examine tumor formation. Tumors usually grow along the lower part of the trachea near the bifurcation, with a solid tumor mass protruding from the surface of the tracheal wall.
15. Measure tumor cross-sectional diameters with Vernier calipers. Determine tumor volume by calculating a spherical body ($\frac{4}{3}\pi r^3$) with the average tumor diameter determined from the square root of the product of the cross-sectional diameters.
16. Tumor samples with adjacent tracheal tissue may be immediately embedded in O.C.T. compound and snap-frozen in liquid $N_2$ for frozen-section preparations for enzymatic staining or immunostaining. Alternatively, the samples can be fixed in 10% formalin for histologic sectioning and analyzed with H&E staining.

3.4.2. Combination Therapy Model

1. Harvest cells at exponential growth phase and suspend cells in PBS at a concentration of $1 \times 10^7$ cells/mL. Store on ice.
2. Irradiate nude mice at 350 cGy of $^{137}$Cs.
3. Subcutaneously inject 0.1 mL of cell suspension into the right flank of mice.
4. Allow tumors to grow for 30–40 d or until the average diameter of tumor reaches 5 mm. (The average diameter is a square root of the product of two perpendicular diameters of tumor measured with calipers.)
5. Inject into each tumor 100 mL of PBS alone or viral solutions ($1 \times 10^{10}$ PFU/mL).
6. Intraperitoneally inject cisplatin solution (3 mg/kg) or equal volume of PBS.
7. Measure tumor size with calipers daily for 2 wk
8. To examine the histologic characteristic of the tumor after treatment, a tumor sample should be collected 3–5 d after treatment. Tumor samples can be immediately embedded in O.C.T. compound and snap-frozen in liquid \( N_2 \) for frozen section preparations (3–5 mm) for enzymatic staining or immunostaining. Alternatively, the samples may be fixed in 10% formalin for histologic sectioning and analyzed with HE staining.

4. Notes

4.1. Determination of Gene Transfer and Expression

1. Generation, propagation, and titer determination of recombinant viruses for experiments should be referred to the appropriate chapters in this book or other references. Adenoviruses need to be purified through CsCl-banding, dialyzed in PBS, and stored in aliquots at \(-80^\circ C\). Retroviruses may be harvested in serum-free medium, filtrated, and stored in aliquots at \(-80^\circ C\).
2. Cell culture medium and conditions should be prepared according to the requirement of the particular cell lines chosen for experiments.
3. For each experiment appropriate controls such as vector and vehicle (mock-infection) controls should be set up.
4. Infectivity of viral vector can also be assessed by using reporter genes such as \( \text{LacZ} \).
5. For vector DNA extraction or Western blot sampling, if cell monolayers need to be harvested or cells are in suspension, pelletize cells by centrifugation. Rinse cells with PBS. Resuspend cell pellets in 100–200 mL of \( H_2O \). Add an equal volume of 2X lysing buffer or 2X SDS-PAGE sample buffer.
6. To semiquantitatively detect the transferred gene and its product in cells, vector-control PCR primers or cellular-protein (b-actin) antibodies can be applied in the same reactions for PCR analysis of the transferred gene or Western analysis of the expressed protein. Two pairs of the PCR primers that detect \( p53 \) gene and adenoviral vector DNA in a single PCR analysis or the anti-\( p53 \) and antiactin antibodies used in the Western blotting have been successfully used in studies of the \( p53 \) adenovirus, as shown in Figs. 2 and 3.
7. To obtain an optimal exposure, the membrane can be exposed serially to X-ray film. In the darkroom, place one side of the X-ray film against the membrane, expose for 1 min, move film toward the other side and expose membrane for 3 min, and so on for 5 min. Develop the exposed film.

4.2. Studies of the Cancer-Inhibitory Effect of the Tumor Suppressor Gene

8. Two types of simple experiments for examining tumor cell growth after transfer of a tumor suppressor gene are described. For further studies, more complicated assays, such as \( [H^3] \) thymidine uptake, growth in soft agar or spheroids, or those that detect specific effects caused by transferred genes, need to be applied.
Dosages of viral vectors for study of the inhibitory effect should be based on the data obtained from infectivity experiments. The MOI that enables 85–95% efficiency of gene transfer should be used.

Trypsinization is one of the key steps that affect the accuracy of cell counts. Condition should be set up to avoid under- or over-trypsinization for harvesting monolayer cells. Alternatively, assays similar to conventional cell counts but that do not acquire trypsin to harvest cells may be applied.

**4.3. Analyses of the Cancer-Inhibitory Mechanisms of Tumor Suppressor Genes**

Cell cycle and apoptosis assays are examples of experiments designed to study the function of the p53 protein. Studies of the mechanisms of cancer inhibition by a given tumor suppressor gene after gene transfer may be specifically designed to meet the needs of the investigators.

Iodide is used to stain cellular DNA. This is a simple and quick method, but it is less accurate than BrdU labeling, which is much more complicated and relatively cumbersome. If more accurate data are needed, BrdU labeling should be used.

Data generated by the Cell Fit program should be analyzed by using the SOBR mathematical model, alternately S-FIT or R-FIT (22) can be used.

For analysis of apoptosis, conventional DNA extraction and gel analysis can be used. Analysis by flow cytometry of cells labeled with fluorescence by TDT reaction is another approach.

If paraffin-embedded tissue sections are used for TDT staining, deparaffinize the tissue sections by treating them with xylene for 5 min twice, 100% ethanol for 5 min twice, 95 and 70% ethanol for 3 min each. Finally, wash slides with PBS for 5 min.

**4.4. Evaluation of Tumor Suppressor Gene Therapy—An Animal Model**

Different tumor cell lines or different tumor suppressor gene vectors need different types of animal models. The two models described in this chapter are for applying recombinant p53 adenovirus in the study of lung cancer treatment in vivo.

Safety procedures should be observed in infecting animals with the viral solutions in the animal room. Use 10% bleach or Bacdown solution to clean the laminar flow hood before and after the operation.

Operations on animals should be handled carefully and delicately to avoid extensive tissue damages and bleeding, particularly when operating on the exposed trachea. To avoid pneumothorax, do not dissect into the upper chest. Since operation on animals by inexperienced personnel often results in animal death, extra animals for each treatment group should be prepared.

In our experiments, a single-pass intratumoral injection of viral solution (100 mL of 1–5 × 10^9 PFU/tumor of a 5-mm average diameter) resulted in 60–70% infection of the tumor mass. The infection rate can be improved by using single-pass injection each day for 3 d.
Tumor Suppressor Genes in Cancer

References


Index

A
AAV, see Adeno-associated virus
Adeno-associated virus (AAV),
genome structure, 25
life cycle, 26, 27
vectors for gene delivery,
adenovirus contamination, 29,
30
history of development, 27
minimal vectors, 27, 28
recombinant vectors,
advantages, 29
efficiency, 29, 30
helper plasmid preparation,
31
packaging systems, 28, 29
plasmid vector preparation,
30
recombinant virus preparation,
cell culture, 30, 31
lipofection, 32, 33, 37, 38
purification, 31, 33, 34
titration by replication
center assay, 31, 32,
35–37
Adenovirus,
cell entry cycle, 5, 6, 8, 135, 136
cystic fibrosis transmembrane
conductance regulator gene
delivery,
advantages, 169
animal models, 171
delivery methods, 171, 172,
176
lung harvesting for histologic
analysis, 176, 177
marker gene detection, 177, 178
materials, 172, 173
transgene-expressed mRNA,
detection by in situ
hybridization,
hybridization reaction, 179
probe generation, 179, 180
ribonuclease contamination,
181
tissue fixation, 178
virus,
desalting of stocks, 174
infection, 180, 181
propagation, 173
purification, 173, 174
strains, 170, 171
titering by gene expression,
174–176
genome, 206
liver-targeted gene transfer,
anesthesia of animals, 208, 209
biliary tract infusion, 210, 211
efficiency, 206
immunologic response against
hepatocytes, 206, 211
laparotomy for portal vein
injection, 209
large animal models, 210
materials, 207
preparation, 207, 208
tail vein injection, 210
p53 gene, adenovirus vector for
cancer therapy,
advantages, 404
animal model evaluation,
combination therapy model,
408, 414, 415
orthotopic lung cancer
model, 408, 414, 416
apoptosis assay, 407, 413, 416
cell cycle analysis, 407, 412,
413, 416
gene expression analysis, 406,
410, 411, 415
infectivity determination, 404,
405, 408, 409
intracellular vector DNA
detection, 406, 409
lung cancer inhibition, 404,
407, 411, 412, 415, 416
preparation, large-scale, 18
receptor-mediated gene delivery,
ablation of infectivity, 8, 9
antibody blocking, 15
asialoorosomucoid-polylysine-
adeno virus-DNA
conjugate,
acid-urea gel electrophoresis
analysis of conjugates, 140
adenovirus conjugation,
144, 145
asialoorosomucoid
preparation, 139
asialoorosomucoid-
polylysine conjugation,
136, 139
asialoorosomucoid-polylysine purification
by acid-urea gel
electrophoresis, 140,
141, 148
asialoorosomucoid-
polylysine purification
by cation-exchange
chromatography, 142,
144
DNA conjugation, 145, 146
gel retardation assay for
DNA concentration
optimization, 145
orosomucoid isolation, 136–
139
conjugates,
adenovirus-polylysine-DNA
complex preparation,
19
combination complexes, 12–
15
design, 17
efficiency assay, 10, 11
instability in vivo, 20
moiety attachment to capsid,
9, 10, 12
poly-L-lysine linkage to
adenovirus, 18, 19
safety, 14, 17
efficiency, in vitro versus in
vivo, 19, 20
endosome escape, 4–7, 136
gene transfer augmentation,
mechanism, 7, 8
nonspecific binding,
minimization, 15, 16
safety of vectors, 206
synovium-targeted gene transfer,
intra-articular injection, 371, 372
materials, 370, 371
transduced synovium, analysis, 372, 373
viral particle number determination, 371

Apoptosis,
aassay, 407, 413, 416
induction by p53, 403
Asialoglycoprotein receptor, see Liver-targeted gene transfer

B
Bone marrow stromal cell,
gene transfer,
cell morphology, 383–385
clinical applications, 375
humans, 380, 383
injection of transduced stromal cells, 383
materials, 377
mice, 379, 380
phenotype uniqueness, 375, 376, 383–385
transplantation, 375, 377

C
Calcium phosphate transfection,
human papillomavirus vector,
121, 122
retroviral packaging cells, 68, 69
Cancer, see Oncogenes; p53 gene;
Tumor cell
CD34, see Hematopoietic stem cells
Central nervous system, diseases and gene therapy vector requirements, 79
CFTR gene, see Cystic fibrosis transmembrane conductance regulator gene
Cisplatin, enhancement of liposome-mediated gene transfer in tumor cells, 330, 335
Cystic fibrosis transmembrane conductance regulator (CFTR) gene,
adeno viral vectors,
advantages, 169
animal models, 171
delivery methods, 171, 172, 176
lung harvesting for histologic analysis, 176, 177
marker gene detection, 177, 178
materials, 172, 173
transgene-expressed mRNA,
detection by in situ hybridization,
hybridization reaction, 179
probe generation, 179, 180
ribonuclease contamination, 181
tissue fixation, 178
virus,
desalting of stocks, 174
infection, 180, 181
propagation, 173
purification, 173, 174
strains, 170, 171
titering by gene expression, 174–176
cystic fibrosis incidence, 169
dysfunction in cystic fibrosis, 153, 169, 170
gene therapy vectors, 153, 154
ion transport in cystic fibrosis, 154, 155
localization in lung cells, 170
retroviral vectors,  
  airway epithelial cells  
    culture, 155, 157, 159, 165, 166  
    isolation, 158  
  assays of gene function,  
    chloride permeability, 164  
    cyclic AMP-mediated chloride permeability, 164  
    efflux assay, 161, 162  
    electrophysiological assay, 162–165  
    materials, 157, 158  
    sodium transport, 164  
  fetal lung delivery,  
    advantages, 187  
    fetal lamb surgery, 188  
    marker gene detection, 189, 190  
    pulmonary toxicity, 191  
    rationale, 185  
    retroviral supernatant production, 188  
    retrovirus administration, 189  
    sheep model, 190  
  gene transfer, 159  
  titer boosting,  
    centrifugal filtration, 160  
    sodium butyrate treatment, 160, 165  
    temperature, 165  
    ultracentrifugation, 161  

D  
  DOTAP transfection, recombinant adeno-associated virus, 33, 37, 38  
  DOTMA/DOPE, applications, 298  

E  
  Electroporation, transfection of retroviral packaging cells, 68, 69, 74  
  Endosome escape, adenovirus, 4–7  

F  
  Fibroblast, tumor vaccine transduction,  
    advantages, 349, 350  
    culture maintenance, 352, 353, 356  
    harvesting fibroblasts for adoptive transfer, 354, 355  
    materials, 350  
    outgrowth of fibroblasts from human skin specimens, 350–352, 355  
    retroviral transduction, 353, 354  
    selection, 354, 356  
    5-Fluorouracil, enhancement of retroviral transduction, 251  
  fos, see Oncogenes  

G  
  Gene gun,  
    clinical applications in skin, 281, 282  
    copy number delivery, 287, 293  
    depth of penetration, 293  
    designs, 282, 283, 285  
    gold particle preparations, 287, 288  
    loading of carrier sheet, 288, 290  
    operational parameters, 285, 286, 290  
    skin treatment, 290, 291  
    tissues transfected, 281, 292  
    transfection efficiency, 293  
    vectors,
coating of particles, 288–290
preparation, 288
size, 287, 292
types, 294
β-Globin, tissue-specific expression with retroviral vectors, 64

**H**

Hematopoietic stem cells (HSC),
abundance in tissues, 249
cycling, 250
developmental hierarchy in mice, 223, 224
enrichment,
anti-CD34 antibodies, 249, 253–255
mice, 227, 228, 235, 236
gene transfer in mice,
committed progenitor colony assays,
bone marrow cell preparation, 231
cell culture, 231, 232, 236
colony identification, 232
gene transfer analysis, 233
efficiency factors, 229, 230
materials, 230, 231
myeloid long-term culture of mouse bone marrow, 233
repopulation assays, 233, 234
retroviral vectors and transduction, 229, 235
growth factors, 224, 250
hematopoiesis, murine models, in vitro,
colony assays for mouse progenitors, 224, 225
long-term bone marrow cultures, 225–236
in vivo assays, 226, 227
maintenance of murine primitive cells, 228, 229
retroviral transduction in humans, amphotropic vector generation, 254, 257
cytokine requirements, 250, 251, 253, 255, 258
efficiency, 252
efficiency assays,
long-term bone marrow culture initiating cells, 257
polymerase chain reaction, 256, 258
Southern blotting, 256–259
5-fluorouracil enhancement, 251
materials, 253
transduction reaction, 255
surface markers, 50
Herpes simplex virus type-1 (HSV-1),
latency-associated transcripts, 80, 83
life cycle, 80, 83
limiting dilution for detection of recombinants,
dot blot analysis, 93, 94
β-galactosidase expression, 91, 92
Southern blot analysis, 93, 94, 97
stocks,
preparation, 94, 95, 97
titration, 94
transfection,
calcium chloride, 91
DNA concentration, 90
DNA isolation, 89, 90, 95, 96
efficiency, 96
vectors,
cytotoxicity removal by gene
deletion,
construction, 85, 86
IE, 83, 84
vhs, 85
suitability for central nervous
system, 79, 80
HSC, see Hematopoietic stem cells
HSV-1, see Herpes simplex virus
type-1
Human papillomavirus (HPV),
genome structure, 117, 118
pathology, 117
replication,
assay, 122
extrachromosomal replication, 117
mechanism, 118, 119
vectors,
delivery systems, 121, 123
design, 122, 123
sequences required for
replication, 119
transfection with calcium
phosphate, 121, 122
viral strains for gene delivery,
120, 121
I
Internal ribosome entry site (IRES),
dicistronic viral vectors, 65,
114, 122, 244
IRES, see Internal ribosome entry
site
K
Keratinocyte, see also Skin
culture,
colony forming efficiency, 270
fibroblast feeder layer, 269,
276, 277
growth rate, 265, 276
materials, 266–268
preparation from biopsy
material, 269, 270
protein secretion, 266, 271, 272
retrovirus-mediated gene transfer,
clinical applications, 266
efficiency assays, 272, 273
grafting of modified sheets,
273–277
infection, 271
virus-producing cell line as
feeder layer, 271
L
Leukemia, see T-cell
Lipofectin, see DOTMA/DOPE
Liposome-mediated gene transfer,
see also DOTMA/DOPE; DOTAP
arterial gene delivery,
efficiency, 128
endothelial cell culture, 129,
130
reporter genes,
alkaline phosphatase, 132
β-galactosidase, 132
serum interference, 133
smooth muscle cell culture,
130
transfection,
in vitro, 131
in vivo, 131, 132
cationic liposomes, types, 127,
298, 299, 329, 330
direct gene transfer, 299
DNA-to-lipid ratio, 298, 299, 332
importance of liposome charge, 128, 297, 298
liposome concentration and efficiency, 133, 334
recombinant adeno-associated virus generation, 32, 33, 37, 38
safety, 128
transfection of retroviral packaging cells, 70
tumor cell delivery, cisplatin enhancement, 330, 335
class I MHC antigen delivery, 299, 300, 302, 304
clinical applications, 297, 298, 330, 331
DNA liposome complex, administration, 302, 304
preparation, 301–303, 330, 335
toxicity, 334
efficiency, 330, 335
gene expression analysis, 303, 304
immune response analysis, 305
liposome selection, 301
plasmid purification, 331, 334
reporter genes, 330, 334
serum inhibition, 334, 335
tissue culture, 331
tumor inoculation on mice, 332
vector production, 300, 301
Liver-targeted gene transfer, adenovirus vectors,
anesthesia of animals, 208, 209
biliary tract infusion, 210, 211
efficiency, 206
immunologic response against hepatocytes, 206, 211
laparotomy for portal vein injection, 209
large animal models, 210
materials, 207
preparation, 207, 208
tail vein injection, 210
asialoglycoprotein receptor, asialoorosomucoid-polylysine-adenovirus-DNA conjugate,
acid-urea gel electrophoresis analysis of conjugates, 140
adenovirus conjugation, 144, 145
asialoorosomucoid preparation, 139
asialoorosomucoid-polylysine conjugation, 136, 139
asialoorosomucoid-polylysine purification, 140–142, 144, 148
DNA conjugation, 145, 146
gel retardation assay for DNA concentration optimization, 145
orosomucoid isolation, 136–139
cell culture, 146, 147
conjugates for gene delivery, 135
expression in hepatocytes, 135
infectivity assay, 147
reporter gene assay, 147, 148
delivery systems, 195
disease candidates, 205
retroviral vectors,
anesthesia of animals, 197, 198
asanguineous perfusion, 196, 199–201
hepatectomy, 196–199, 202
recombinant virus production, 196, 197, 202
transgene expression analysis, polymerase chain reaction, 201, 202
tissue fixation, 201
Lung, see Cystic fibrosis
transmembrane conductance regulator gene
M
MHC class I molecule, tumor vaccine, 299, 300, 302, 304, 309, 310
Murine leukemia virus, gene transfer vectors,
components, 61
design, 60, 61, 65
insert size, 59
receptor binding, 60
stability, 59, 60
O
Oncogenes

fos,
inhibition of expression, 392
ribozyme,
assays of efficacy, 393–398
cell entry, 392
design, 392
effects on cell morphology, 396, 397
induction, 397
transfection of tumor cells by electroporation, 393, 398
mechanisms of action, 391
types, 391
P
p53 gene,
adeno virus vector for cancer therapy,
advantages, 404
animal model evaluation,
combination therapy model, 408, 414, 415
orthotopic lung cancer model, 408, 414, 416
apoptosis assay, 407, 413, 416
cell cycle analysis, 407, 412, 413, 416
gene expression analysis, 406, 410, 411, 415
infectivity determination, 404, 405, 408, 409
intracellular vector DNA detection, 406, 409
lung cancer inhibition, 404, 407, 411, 412, 415, 416
apoptosis induction, 403
mutation and cancer, 403
Poliovirus,
capsid processing, 103
foreign protein expression, gene subcloning, 108
insert size, 105
processing, 105, 106, 108
infectivity, 114
receptor, 103
replicon,
encapsulated replicon detection, 111, 112
removal of vaccinia virus, 112, 113
serial passage, 110, 114
structure, 103, 105, 106

titer assay, 113, 114

transfection, 109

template,

preparation, 108, 109

transcription, in vitro, 109
tissue culture, 108

vaccinia virus expressing P1 as

helper virus, 106, 112, 113

R

Receptor-mediated endocytosis,

adenovirus facilitation, see

Adenovirus

advantages in gene delivery, 2
DNA conformation, 2
hepatocyte targeting, 2, 3
vector,

components, 1, 2

efficiency, 4

Replication center assay, adeno-

associated virus, 31, 32, 35–37

Retroviral gene transfer,

constitutive internal promoter, 62, 64
cystic fibrosis transmembrane

conductance regulator gene

transfer,

airway epithelial cells,
culture, 155, 157, 159, 165, 166

isolation, 158

assay of gene function,

chloride permeability, 164
cyclic AMP-mediated

chloride permeability, 164
efflux assay, 161, 162

electrophysiological assay,

162–165

materials, 157, 158

sodium transport, 164

fetal lung delivery,

advantages, 187

fetal lamb surgery, 188

marker gene detection, 189, 190

pulmonary toxicity, 191

rationale, 185

retroviral supernatant

production, 188

retrovirus administration, 189

sheep model, 190
gene transfer, 159
titer boosting,

centrifugal filtration, 160

sodium butyrate treatment,

160, 165
temperature, 165

ultracentrifugation, 161
dicistronic vectors, 65
dual-promoter vectors, 64
fetal tissues in experimental

animals, 186–188

fibroblast infection, 50

helper-free retrovirus packaging

cells,

293 cells,

cell growth, 47, 48, 51, 52
chloroquine treatment, 53
freezing, 48, 51, 52
infection of nonadherent
cells by cocultivation,

51, 54, 55
retroviral supernatant,

harvesting, 50

safety, 51, 52

screening of clones, 45, 46, 52, 55
T/17 clone, 45
thawing, 48
transfection, 48–50, 53, 54, 56
vesicular stomatitis virus G-glycoprotein pseudotyping, 46, 47, 51
culture, 67, 68
infectious titer, 42
kat system, 44
screening of clones, 42, 43
transient retroviral production, 43, 44
ψ2 cells, 41
helper virus detection by mobilization assay, 72
hematopoietic stem cells, gene transfer,
humans,
amphototropic vector generation, 254, 257
cytokine requirements, 250, 251, 253, 255, 258
efficiency, 252
5-fluorouracil enhancement, 251
long-term bone marrow culture initiating cells, 257
materials, 253
polymerase chain reaction assay, 256, 258
Southern blotting, 256–259
transduction reaction, 255
mice,
committed progenitor colony assays, 231, 232, 236
efficiency factors, 229, 230
gene transfer analysis, 233
materials, 230, 231
myeloid long-term culture of mouse bone marrow, 233
repopulation assays, 233, 234
retroviral vectors and transduction, 229, 235
infection efficiency optimization, cocultivation of target and producer cells, 73, 74
cross-infection and ping-pong, 73
virus concentration, 73, 74
keratinocyte gene transfer, clinical applications, 266
efficiency assays, 272, 273
grafting of modified sheets, 273–277
infection, 271
virus-producing cell line as feeder layer, 271
liver-targeted gene transfer, anesthesia of animals, 197, 198
asanguineous perfusion, 196, 199–201
hepatectomy, 196–199, 202
recombinant virus production, 196, 197, 202
transgene expression analysis, polymerase chain reaction, 201, 202
tissue fixation, 201
long terminal repeat-driven expression, 61, 62
murine leukemia virus vectors, components, 61
design, 60, 61, 65
insert size, 59
receptor binding, 60
stability, 59, 60
pantropic retroviral vectors and
T-cell targeting,
cell growth, 215, 216
concentration of virus, 216,
219, 221
detection of viral DNA, 216,
220, 221
generation in 293 cells, 216–
219
gene therapy of leukemia, 214,
215
infection of T-cells, 216, 219,
220
stability, 213, 214
recombinant virus titer,
geneneration of high-titer
producers, 65–67, 73
requirements, 41
T-cell gene transfer,
assay of transfer, 243, 245,
246
factors controlling, 241, 242
infection,
efficiency, 242, 243, 246
protocol, 245
vector design, 244
tissue-specific expression, 64
transfection of packaging cells,
calcium phosphate, 68, 69
electroporation, 68, 69, 74
lipofection, 70
markers, 68
screening of clones,
fluorescence-activated cell
sorting, 71, 72
proviral DNA analysis, 71
RNA dot blot analysis, 70
selectable marker
expression, 72
titration on fibroblasts, 70,
71
tumor cells,
advantages, 308
cell lines,
construction, 315
producer maintenance, 316–
318
tumor maintenance, 317,
319
cytokine secretion assay, 317,
321–323
gene transfer, 317, 319–321
rationale for gene delivery,
312, 313
retroviral supernatant
preparation, 316, 318,
319
structure of vector, 314, 315
Rheumatoid arthritis,
antiarthritic proteins, 358
gene therapy, see Synovium-
targeted gene transfer
pharmaceutical therapy, 357
Ribozyme,
cell entry, 392, 398
design, 392, 398
fos ribozyme,
assays of efficacy, 393–398
effects on cell morphology,
396, 397
induction, 397
transfection of tumor cells by
electroporation, 393, 398
hammerhead ribozymes, 397
specificity, 398
S
Skin, *see also* Keratinocyte,
advantages as gene transfer
tissue, 292, 293
particle-mediated gene transfer,
clinical applications, 281, 282
copy number delivery, 287, 293
depth of penetration, 293
gene gun,
designs, 282, 283, 285
operational parameters, 285, 286, 290
gene transfer, 291
gold particle preparations, 287, 288
immune response, 294
loading of carrier sheet, 288, 290
reporter genes, 281, 289, 291, 293
skin treatment, 290, 291
transfection efficiency, 293
vectors,
coating of particles, 288–290
preparation, 288
size, 287, 292
types, 294

Sodium butyrate, boosting of
retroviral titers, 160, 165

Stromal cell, *see* Bone marrow
stromal cell

Synovium-targeted gene transfer,
adenovirus vector,
 intra-articular injection, 371, 372
materials, 370, 371
transduced synovium, analysis, 372, 373
viral particle number
determination, 371
ex vivo approach,
cell culture, 360, 362, 363, 365
harvesting of tissue, 360, 362, 365
materials, 361, 362
passage of synoviocytes, 360, 363, 366
post-transplantation testing,
364–366
transduction reaction, 360, 361, 363, 366
transgene expression assays, 361, 363, 364
transplantation of transduced
synoviocytes, 358–361, 364, 366
intra-articular injection,
transduced synoviocytes, 358–361, 364, 366
vector, 358, 371, 372
synoviocytes,
protein secretion, 369, 370
types, 359, 369

T
T-cell,
pantropic retroviral vectors,
cell growth, 215, 216
concentration of virus, 216, 219, 221
defined, 213
detection of viral DNA, 216, 220, 221
gene therapy of leukemia, 214, 215
generation in 293 cells, 216–219
infection of T-cells, 216, 219, 220
stability, 213, 214
retrovirus-mediated gene transfer,
assay of transfer, 243, 245, 246
factors controlling, 241, 242
infection,
efficiency, 242, 243, 246
protocol, 245
vector design, 244
tumor cell antigen recognition,
308, 309
Transferrin,
efficiency of cellular
internalization, 1
receptor-mediated endocytosis in
gene delivery, 3, 4
Tumor cell,
antigen recognition by T-cells,
308, 309
cytokines,
antitumor activity, 339, 344, 345
cell proliferation assay, 340, 343
cytotoxicity assay, 341, 344
detection in modified cells,
317, 321–323, 341, 343, 344
fibroblast transduction,
advantages, 349, 350
culture maintenance, 352, 353, 356
harvesting fibroblasts for
adoptive transfer, 354, 355
materials, 350
outgrowth of fibroblasts
from human skin
specimens, 350–352, 355
retroviral transduction, 353, 354
selection, 354, 356
murine cell lines for gene
transfer, 342
vaccine effect, analysis of
modified tumor cells,
344–346
 fos ribozyme,
assays of efficacy, 393–398
effects on cell morphology,
396, 397
induction, 397
transfection of tumor cells by
electroporation, 393, 398
genetic approaches for
augmenting antitumor
immune responses, 307
liposome-mediated gene transfer,
cisplatin enhancement, 330, 335
class I MHC antigen delivery,
299, 300, 302, 304
clinical applications, 297, 298, 330, 331
DNA liposome complex,
administration, 302, 304
preparation, 301–303, 330, 335
toxicity, 334
efficiency, 330, 335
gene expression analysis, 303, 304
immune response analysis, 305
liposome selection, 301
plasmid purification, 331, 334
reporter genes, 330, 334
serum inhibition, 334, 335

tissue culture, 331
tumor inoculation on mice, 332
vector production, 300, 301

p53 gene, adenovirus vector for cancer therapy,
advantages, 404
animal model evaluation,
combination therapy model, 408, 414, 415
orthotopic lung cancer model, 408, 414, 416
apoptosis assay, 407, 413, 416
cell cycle analysis, 407, 412, 413, 416
gene expression analysis, 406, 410, 411, 415
infectivity determination, 404, 405, 408, 409
intracellular vector DNA detection, 406, 409
lung cancer inhibition, 404, 407, 411, 412, 415, 416
retrovirus-mediated gene transfer, advantages, 308
cell lines,
construction, 315

producer maintenance, 316–318
tumor maintenance, 317, 319
cytokine secretion assay, 317, 321–323
gene transfer, 317, 319–321
rationale for gene delivery, 312, 313
retroviral supernatant preparation, 316, 318, 319
structure of vector, 314, 315
tumor vaccines,
adjuvants, 346
history of development, 309, 310, 339
limitations, 307, 308, 340, 349
murine studies, 310–312

Tumor suppressor genes, see p53 gene

V
Vaccine, see Tumor cell
Vesicular stomatitis virus, G-glycoprotein pseudotyping of retrovirus, 46, 47, 51, 213