Assessment of Recombinant Adenoviral Vectors for Hepatic Gene Therapy

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ABSTRACT

Recombinant adenoviral vectors have recently been used to transfer genes into a number of different cell types in vitro and in vivo. A recombinant adenoviral vector bearing the Escherichia coli β-galactosidase (β-gal) gene was used to quantitate the frequency of hepatocyte transduction in the mouse after direct viral infusion into the portal vein. When 10^{10} adenoviral particles were infused, over 95% of the hepatocytes were transduced in vivo as determined by x-gal staining. The transduction protocol is relatively safe in that there is no detectable helper virus production in transduced animals and that very few extrahepatic cells are transduced by this method. There is also no evidence of significant liver pathology unless substantially greater quantities of virus are used. However, the transduced hepatocytes do not appear to persist in vivo because the percentage of hepatocytes expressing β-gal declined over time. Four months after the procedure, 0.5–10% of the hepatocytes contain detectable β-gal activity in vivo. The change in β-gal-positive cells correlates with decreasing amounts of adenoviral DNA. Thus, current recombinant adenoviral vectors may have clinical applications in gene therapy for acute hepatic disorders.

OVERVIEW SUMMARY

This study demonstrates that the recombinant adenovirus can be used to transfer genes into greater than 95% of mouse hepatocytes in vivo. The expression from the majority of transduced hepatocytes is slowly lost over a 3- to 4-month period. This vector system has the advantage of being a very efficient means of gene transfer into the liver; the disadvantage of the system relates to the transient nature of expression in most of the transduced hepatocytes. Further experimentation will be required to determine whether a significant number of transduced hepatocytes will remain indefinitely.

THE ADENOVIRUS has recently been developed as an efficient means of gene transfer into a number of epithelial-derived tissues (Gilardi et al., 1990; Stratford-Perricaudet et al., 1990; Quantin et al., 1992; Rosenfeld et al., 1991, 1992). Deletions in the viral 35-kb genome have been made in the E3 and E1 regions so that recombinant gene constructs can be inserted into this vector. Because the adenovirus has a natural tropism for the lung epithelium, there has been extensive interest in using the vector for gene transfer into this tissue for a variety of genetic disorders that include α1-antitrypsin deficiency (Gilardi et al., 1990) and cystic fibrosis (Rosenfeld et al., 1992). Recombinant adenoviral vectors have the advantage of being able to transduce nonproliferating cells and taken together with the ability to

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produce purified high-titer virus, this vector system offers an attractive alternative to the more commonly used retroviral vectors. Previously studies (Stratford-Perricaudet, 1990) involving adenoviral gene delivery to the liver clearly demonstrate that mouse hepatocytes can be transduced in vivo. A recombinant adenovirus encoding the ornithine transcarbamylase (OTC) cDNA has been transferred to sparse fur (Spf) mice; enzyme activity was present in the liver of recipients for various durations. Adenoviral-mediated transfer of the OTC cDNA resulted in the phenotypic correction of the Spf ash animals. Recently, Jaffe et al. (1992) demonstrated human α1-antitrypsin production from rat liver after transduction with a recombinant adenoviral vector in which only 1% of hepatocytes were transduced in vivo. To assess the suitability of recombinant adenoviral vectors for hepatic gene therapy, we have used the mouse as a model for direct adenoviral-mediated gene transfer to hepatocytes in vivo.

METHODS

Animals

Female C57Bl/6 mice, ages 3–5 weeks, were used in the described experiments.

Adenovirus production

The recombinant adenoviral vector Ad.RSVBgal containing β-galactosidase (β-gal) under the transcriptional control of the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter is a replication-deficient human adenovirus that has been described previously (Stratford-Perricaudet et al., 1992). Adenovirus was prepared as described (Graham and Prevec, 1991) and purified by two rounds of cesium chloride ultracentrifugation. The purified virus was dialyzed in 10 mM Tris HCl, pH 7.4, 1 mM MgCl2 and stored at 4°C for immediate use. Viral titers were determined by O.D. (particles per ml) and by plaque assay (Graham and Prevec, 1991). In most experiments, the plaque titer was within 1 log of the O.D. titer.

Surgical manipulation

Mice were anesthetized with avertin (Kay et al., 1992b). Portal vein infusion was performed via a 27- or 30-gauge catheter over about 1 min. The total infusate was about 100 μl. After the infusion was completed, gel foam was applied to the portal vein at the site of infusion. Control animals were either infused with phosphate-buffered saline (PBS) or another adenoviral vector that does not encode β-gal.

Hepatocyte isolation and culture

Mouse hepatocyte isolation and culture was as previously described (Ponder et al., 1991). X-gal staining was performed 6–12 hr after isolation and blue cells were determined 16 hr later. For the in vitro studies, 2 × 10⁵ hepatocytes were infected for 1 hr in a six-well dish with adenovirus in a total volume of 200 μl after serial dilution in PBS. X-gal staining was performed 48 hr later.

Histology

Tissues were excised at the time of sacrifice and embedded in O.C.T and immediately frozen in 2-methylbutane cooled by liquid nitrogen and stored at −80°C until they were sectioned and stained with X-gal (Ponder et al., 1991). For routine hematoxylin and eosin staining, the tissues were fixed in 10% formalin prior to sectioning and staining.

Helper virus assays

Supernatants from isolated hepatocytes were collected for 48–72 hr and used to infect either Hepa la cells (mouse hepatoma) or 293 cells for a plaque assay as described above. Similar experiments were performed using freshly isolated mouse serum. The Hepa la cells were stained with X-gal 48 hr later. Plaques were counted 7–10 days after infection.

DNA blot analysis

Whole liver DNA was prepared from selected animals (Ponder et al., 1991) and 20 μg was digested with Hind III. The DNA was loaded onto a 0.7% agarose gel and following electrophoresis was transferred to a Hybond (Amersham) filter. The blot was hybridized sequentially with a radiolabeled 3.5-kb β-gal DNA fragment and a 2.2-kb Hind III/Xba I mouse α1-anti-trypsin exon 4 probe in rapid hybridization buffer (Amersham) according to the manufacturer’s instructions. The blot was washed in 0.1 × SSC at 65°C and exposed to X-ray film.

RESULTS

Quantitative transduction of primary mouse hepatocytes in vitro

The transduction frequency of recombinant adenovirus in hepatocytes was quantitatively determined in vitro. Various concentrations of the Ad.RSVBgal virus were used to infect primary hepatocyte cultures, followed by X-gal staining of the cells 48 hr later. The proportion of blue cells is a measure of the transduction frequency (Table 1). At a multiplicity of infection (moi) of 100, 95–100% of the hepatocytes were reproducibly transduced. When the moi was increased by factors of 10–100, the cytopathic effect (CPE) of the virus became evident when most of the cells were dead within hours. Lower moi resulted in proportionally reduced transduction frequencies. Hepatocytes infected with PBS or control adenoviruses did not stain blue. Thus, it is possible to transduce primary mouse hepatocytes quantitatively in culture using recombinant adenoviral vectors.

Quantitative transduction of mouse hepatocytes in vivo

To determine the maximal number of hepatocytes that can be transduced with the recombinant adenoviral vector in vivo, mice were injected with different quantities of the viral vector via the portal vein. One week later, animals were sacrificed and liver sections were stained with X-gal. Extensive staining of the hepatocytes are shown in Plates A and B. Because the β-gal
gene contains the SV40 nuclear localization sequence. X-gal staining is localized to the nucleus. To assess the transduction efficiency in vivo more accurately, hepatocytes were isolated from the virus-infused animals and the percent of transduced cells was calculated by counting the number of blue cells in a population of cells derived from the whole liver, ensuring the observed transduction frequency represents that of the entire organ (Plate A). For each animal, at least 500 cells were counted. When $1 \times 10^5$ viral vectors were infused, 95–100% of the hepatocytes were transduced in vivo. (Plate A and Fig. 1). Small pieces of frozen tissue were obtained for X-gal staining from animals that underwent hepatocyte isolation and culture following adenovirus infusion. Although 95–100% of the hepatocytes stain blue in culture, only 60–75% of the nuclei stain blue in the hepatocyte sections (Plate B). Because there are about $1 \times 10^5$ hepatocytes in the mouse, the moi was about 100. The proportion of blue cells fell when less virus was infused. When 20-fold less virus was used, only about 5–10% of the hepatocytes were transduced (Fig. 1). However, when $7 \times 10^5$ viral vectors were infused (moi about 700), most of the animals died within the first several days. The surviving animals also had transduction rates of 95–100% (data not shown).

**Absence of helper virus production in vivo**

To determine whether the high transduction frequency in vivo was the result of viral replication in vivo, helper virus assays were performed. The assay consisted of isolating hepatocytes 2 weeks after gene transfer in vivo in 4 animals. Half of the cultured cells were stained with X-gal, while the other half were kept in media for an additional 2 days. Over 98% of the hepatocytes stained blue. The conditioned media from transduced hepatocytes was transferred to hepatoma cells or 293 cells. The hepatoma cells were stained 48 hr later with X-gal and the 293 cells were analyzed for the formation of plaques. In all animals studies, there were no blue hepatoma cells and no plaque formation was detected on 293 cells (data not shown). Similar results were obtained when the serum was used in place of conditioned media. Taken together, there was no evidence suggestive of infectious virus production from transduced liver cells.

**Pathological analysis of liver after viral transduction**

Cytotoxic effects have been described in host cells that are infected with adenovirus; however, because wild-type adenovirus rarely infects liver cells, it is not known whether the recombinant vectors would produce extensive hepatocellular damage in vivo after viral infusion. Animals were infused with $1–7 \times 10^5$ particles as described above and sacrificed 2 or 8 days later. Portions of the liver were either fixed in formalin for routine histopathologic examination or frozen for X-gal staining. The frozen sections were stained with X-gal as a control to confirm that the appropriate proportion of hepatocytes were transduced as expected from the data in Fig. 2. When $1 \times 10^5$ viral particles were infused, there was no apparent liver pathology 2 or 8 days after the transduction procedure (Plates C and D). However, when $7 \times 10^5$ viral particles were infused, there was evidence of patchy hepatic necrosis 2 days later (Plate D) in the surviving animals; no further necrotic changes were observed in these animals at 8 days (not shown). As mentioned previously, most of the animals that were infused with $7 \times 10^5$ particles died. Because these animals were not available at the time of death, it is anticipated that a lethal dose of adenovirus would lead to greater hepatic necrosis. The data presented above suggest that enough adenovirus can be safely infused to transduce most of the hepatocytes quantitatively but that when excessive amounts of virus are used lethal liver damage may occur.

The adenovirus can infect a number of different cell types and the delivery system used here does not preclude the infection of other tissues and cell types. To evaluate whether other tissues are transduced by this gene delivery system, tissues from an animal were examined after gene transfer for the expression of β-gal by X-gal staining. The tissues that expressed at least one to 20 blue cells within a section containing multiple thousands of cells included the spleen, small intestine, stomach, muscle, heart, lung, and pancreas. Tissue that were examined and showed no staining included the brain, thymus, ovary, and adrenal glands. Although adenovirus can infect many cell types, using the portal vein as the route of delivery the first pass through the liver results in the vast majority of the virus being taken up by the hepatocytes.

**Persistence of gene expression from transduced hepatocytes in vivo**

The experiments described above clearly demonstrate that the majority of hepatocytes can be transduced with adenovirus without causing significant tissue injury. The next question we addressed is longevity of expression after adenoviral-mediated gene transfer. In separate experiments, animals were transduced with $1 \times 10^5$ viral particles via the portal vasculature. Some of the animals were sacrificed within the first 2 weeks to determine transduction efficiencies of hepatocytes by total hepatocyte isolation followed by X-gal staining. The rest of the animals were sacrificed at different time points after viral infusion (Fig. 2). The relative proportion of transduced cells decreased over time. By 14–16 weeks, the transduced hepatocytes represented only 0.5–10% in individual animals.

To determine whether the decline in gene expression is due to loss of DNA, animals that were injected with $1 \times 10^5$ adeno-
viral particles were sacrificed at 5 days and 4.5 months post-infusion. Portions of the liver were stained with X-gal to estimate the in vivo transduction frequency. The results were essentially the same as in Fig. 2. DNA was extracted from the remaining liver and digested with Hin d III. A DNA blot was sequentially hybridized with a radiolabeled β-gal probe (Fig. 3) and a mouse hAAT DNA probe (data not shown). The latter probe showed equal hybridization signals in all lanes. The hybridization signal in the 4.5-month animals (Fig. 3, lanes b–e) are about 10-fold less than the 5-day animals (Fig. 3, lanes f–i), a result that is consistent with the number of transduced cells determined by β-gal staining. The number of adeno viral genome copies was estimated by adding different genome equivalents restriction digested β-gal plasmid DNA (Fig. 3, lanes k and l). The 4.5-month animals have less than one adeno viral copy per genome, whereas the 5-day animals have about five copies per genome. Thus, the decline in β-gal-positive cells roughly correlates with the amount of adeno viral DNA.

**DISCUSSION**

A number of methods have been developed for the delivery of genes to the liver. Our laboratory (Kay et al., 1992a) and others (Chowdhury et al., 1991) have developed an ex vivo approach in animals model that involves isolation of hepatocytes after partial hepatectomy, followed by in vitro gene transduction and autologous transplantation of genetically reconstituted cells into the animals. More recently, it has been reported (Ferry et al., 1991; Kaleko et al., 1991) that hepatocytes can be transduced in vivo by direct injection of recombinant retroviral vectors after partial hepatectomy. We have also demonstrated expression of human α1-antitrypsin for at least 6 months in mice after direct viral injection into the portal vein (Kay et al., 1992b). Although the retroviral vectors have been successful in hepatic gene delivery, they involve invasive surgical procedures, including a 70% partial hepatectomy prior to recombinant virus infusion. These methods of gene transfer have resulted in a relatively low frequency of hepatocyte transduction in vivo, with the best conditions resulting in gene transfer in about 2% of hepatocytes.

A variety of cell types including hepatocytes (Levreo et al., 1991; Jaffe et al., 1992; Stratford-Perricaudet, 1992) have been demonstrated to be efficiently transduced by recombinant adeno viral vectors in vitro. We have carefully quantitated the ratio of adeno virus to cultured mouse hepatocytes necessary to transduce all hepatocytes in vitro. When 100 viral particles/cell were used, we were able to transduce over 95% of the cells. Much higher concentrations of adeno virus lead to cell death within hours, a result that is probably related to the toxicity of the viral proteins rather than replication of virus.

Although Jaffe et al. (1992) were able to demonstrate quantitative transduction of rat hepatocytes in vitro, they were able to transduce only about 1% of hepatocytes when the virus was delivered in vivo via the portal vasculature. In this study, virtually all hepatocytes could be transduced in vivo. The difference in transduction frequencies in our study and Jaffe et al. (1992) may be related to inherent differences in the ability of human adeno virus to transduce hepatocytes in vivo between the two species. Because both rat and mouse hepatocytes can be transduced at high frequencies in vitro, the mechanism account for differences is not clear.

The portal vein infusion methods that lead to quantitative transduction of hepatocytes illustrated that the virus is hepatotropic by this route of delivery in that very few cells in other tissues were transduced with the vector. Furthermore, enough adenovirus could be delivered to mice to transduce all the hepatocytes quantitatively without histopathologic evidence of liver injury. When excessive amounts of virus were used, however, the mortality rate was substantially increased. The animals that did survive excessive adeno viral transduction showed transient evidence of hepatic necrosis. Taken together with the absence of detectable wild-type activity from isolated hepatocytes that had been transduced in vivo, adenovirus can be infused to transduce most of the hepatocytes in vivo without apparent toxicity to the animal.

Although gene transfer to mouse hepatocytes is very efficient with this vector, the number of transduced cells appears to decrease over time. Although it is not practical to follow individual animals over time using an intracellular marker such as β-gal, the reproducibility of transduction efficiency allowed the evaluation of individual animals at different time points after gene delivery. At 3–4 months, 0.5–10% of hepatocytes still express β-gal as determined by X-gal staining. The decrease in β-gal-positive cells correlates with the decrease in adeno viral genome copy. It is not clear whether or not the animals will continue to contain and express recombinant adeno viral DNA at a lower frequency or whether all of the β-gal activity will eventually be lost in all cells. The adeno viral genome does not integrate into the host chromosome (Stratford-Perricaudet et al., 1992), and, as a result, the DNA may be gradually lost from cells over time. It is unlikely that transduced hepatocytes are selectively killed in vivo, because with a transduction efficiency of near 100% there should be evidence of hepatocyte regeneration in transduced animals, which was not seen in histopathologic sections. These important issues will need to be addressed experimentally.

In summary, although the recombinant adeno viral vector used in these studies represents a very efficient method of...
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FIG. 1. Dose-response of adenovirus-mediated gene transduction of hepatocytes in vivo. Different amounts of Ad.RSV β-gal adenoviral particles were infused into the portal vein. One week later, hepatocytes were isolated and stained with X-gal. The proportion of β-gal-positive cells were determined by counting random fields. Each point represents a single animal.

FIG. 2. Longevity of gene expression after adenovirus-mediated gene transfer. Animals were infused with the Ad.RSVBgal adenoviral vector and hepatocytes were isolated at different times after infusion. The proportion of β-gal-positive cells was determined by counting random fields for cells containing blue nuclei. Each point represents a single animal.
FIG. 3. Adenoviral DNA determinations after hepatic gene transfer in vivo. Animals were infused with $1 \times 10^{10}$ Ad.RS VBgal particles and their livers were removed at 5 days (lanes f–i) or 4.5 months (lanes b–e) after viral infusion. Twenty micrograms of total DNA that was digested with Hind III was subjected to Southern blot analysis. The blot was hybridized with a $^{35}$P-labeled $\beta$-gal gene fragment. Lane a represents DNA from a control animal. Lanes j and k represent 30 pg (1 genome equivalent) and 150 pg (5 genome equivalents) of Hind III-digested $\beta$-gal plasmid DNA mixed with control Hind III-digested mouse genomic DNA. The hybridization signal represents a 3.5-kb fragment.

Hepatic gene delivery in vivo, persistence of expression decreases over time. Clinical applications include the use of these vectors for short-term therapy of acute disorders. The possibility of repetitive treatments for extended therapies of chronic diseases should also be investigated.

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