Hepatic Gene Therapy: Persistent Expression of Human α1-Antitrypsin in Mice after Direct Gene Delivery In Vivo

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ABSTRACT

The liver represents an excellent target organ for gene therapy. The current strategy for hepatic gene therapy involves the isolation of primary hepatocytes from a resected liver lobe, transduction of therapeutic genes in vitro followed by autologous hepatocellular transplantation. This ex vivo approach is a rather complex procedure in its entirety; thus, a simple method for direct gene delivery into hepatocytes in vivo has been developed. The procedure involves partial hepatectomy followed by the portal vein infusion of recombinant retroviral vectors. Histological analysis of hepatocytes after in vivo delivery of a recombinant retrovirus bearing the E. coli β-galactosidase gene showed that 1–2% of the parenchymal cells were transduced. Direct hepatic transfer of human α1-antitrypsin cDNA under the transcriptional direction of the albumin promoter–enhancer led to constitutive expression of the human protein in the sera of recipients at concentrations of 30–1,400 ng/ml for at least 6 months. The experimental animals showed no signs of illness and histologic analysis of the liver revealed no evidence of pathologic abnormalities. The results suggest that the in vivo approach is an attractive alternative for hepatic gene therapy.

OVERVIEW SUMMARY

The liver represents a major target organ for gene therapy. A simple method for direct retroviral-mediated gene delivery into mouse hepatocytes in vivo was developed. By using a vector that encodes a human serum protein marker it was possible to demonstrate long-term constitutive expression in the sera of recipients. This approach represents an attractive alternative to the complex ex vivo strategy that is currently being used for hepatic gene therapy.

INTRODUCTION

A NUMBER OF DIFFERENT delivery systems have been developed to transfer genes into the cells of living animals. To date, recombinant retroviral vectors are the only gene transfer system that have been shown to integrate permanently into host chromosomal DNA. Our laboratory and others have focused on the delivery and expression of exogenous genes from the liver and have developed the technologies for the transplantation of hepatocytes in mice such that the donor cells reside in the liver parenchyma and function as hepatocytes for the life of the recipient (Gupta et al., 1991; Ponder et al., 1991). More recently, we and others have combined the two technologies and developed canine and rabbit models for the autologous transplantation of hepatocytes after retroviral-mediated gene transfer (Chowdhury et al., 1991; Kay et al., 1992). This method of hepatic gene transfer into animals is technically complex and labor intensive. Thus, we have investigated alternate methods of hepatic gene delivery using retroviral vectors.

Recently, Kaleko et al. (1991) demonstrated that retroviral vectors could be transduced into liver cells in vivo by direct injection of virus into the parenchyma after carbon tetrachloride treatment. Ferry et al. (1991) have demonstrated transduction of retroviral vectors into rat hepatocytes after partial hepatec-

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tomy followed by a complicated surgical procedure that involves isolated perfusion of the liver with a viral vector. In the later study, transduced hepatocytes were found at 6 months after gene transduction. In both studies, the level of transgene expression in the individual recipient animals over time was not addressed. If these techniques are to be used clinically for therapeutic purposes, long-term constitutive expression in individual recipient animals must be demonstrated. Here we describe a simple method for direct retroviral-mediated gene transduction of mouse hepatocytes in the liver using a vector that encodes the intracellular Escherichia coli β-galactosidase marker. Using a second viral vector that encodes the human serum protein α1-antitrypsin, constitutive expression of the human protein was detected in the sera of recipient animals for 6 months. This method may be applicable for the permanent correction of genetic deficiency states such as the hemophilias or hepatic enzyme deficiencies.

MATERIALS AND METHODS

Construction of retroviral vectors

The construction of the LNCM-hAAT vector has been previously described (Kay et al., 1992). The hAAT cDNA was cloned into the Eco RI site of pBluescript (PBS-hAAT). The Hind III I fragment of pAN/T2-NB containing the 2.8-kb albumin promoter–enhancer (K. Zaret, personal communication) was cloned into the Xba I and blunt Spe I sites of PBS-hAAT such that this fragment is 5' to the hAAT cDNA (pAlb-hAAT). The albumin promoter was derived from the 800-bp fragment in plasmid pAT2 (Zaret et al., 1988) and the 2-kb albumin enhancer was derived from the genomic Nhe I–Bam HI fragment (Pinkert et al., 1987). The albumin promoter–enhancer/hAAT cDNA fragment was removed from pAlb-hAAT by cutting and blunting the Not I site followed by Cla I digestion. This 4.2-kb fragment was cloned into the LNCX vector (Miller and Rosman, 1989) after the CMV promoter was removed (Bam HI–Cla I fragment). The Bam HI site was blunt and the albumin promoter–enhancer/hAAT cDNA fragment was then ligated into this vector producing pLNAIb-hAAT.

The blunted Xho I and Not I 4.2-kb fragment from pAlb-hAAT was cloned into the blunted Xho I site of pGEN” (Soriano et al., 1991) vector to produce the pGenAlb-hAAT plasmid. The CMV promoter contained in the LNCX vector plasmid was replaced with the PGK promoter (unpublished data) to produce the pLNpX plasmid. The neomycin phosphotransferase gene was replaced with the β-galactosidase (Friedrich and Soriano, 1991) minigene by blunt-end ligation into the Bcl I–Bam HI site to produce pLBGPgk. The retroviral vector–containing plasmids were transfected into GP+E–86 (Markowitz et al., 1988a and/or GP+AM12 (Markowitz et al., 1988b) packaging lines and high-titer clones were selected as previously described (Kay et al., 1992). Titration of LNAIb-hAAT, LNCM-hAAT, and LBGPgk were performed on rat 208F cells by methods described by Lynch and Miller (1991). The titer of the GenAlb-hAAT vector was estimated by limiting dilutions of this virus and the LNCM-hAAT virus on rat 208F cells followed by Southern blot analysis using a radiolabeled hAAT cDNA probe. The signal for the GenAlb-hAAT was about 50-fold lower than that for LNCM-hAAT.

Helper virus assay

A marker rescue assay was performed on supernatants from each viral clone as described (Belmont et al., 1988). Additionally, each animal that underwent gene transfer procedures for long-term expression studies were studied for the production of replication-competent virus by using fresh serum (60 μl per animal) 3–6 months after gene transfer in the same marker rescue assay. No replication-competent virus was detected in both sets of experiments.

Cell lines and hepatocyte isolation

The rat embryo fibroblast 208 F cell lines were grown in high glucose DMEM containing 10% heat-inactivated hyclone, 1 mM glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin. Mouse hepatocytes were isolated by the collagenase perfusion method and cultured by methods that have been previously described (Ponder et al., 1991). X-gal histochemical staining of cultured hepatocytes and liver tissue was as previously described (Ponder et al., 1991).

Partial hepatectomy and retroviral transduction in vivo

C57Bl6 female mice ages 4–5 weeks weighing 10–12 grams were anesthetized with an intraperitoneal administration of 0.5 ml of 20 mg/ml Avertin (2,2,2-tribromoethanol). A 1-cm midline abdominal incision extending from the xiphoid process was made after sterile preparation. The right lateral, median, and left lateral lobes were sequentially ligated with a 5-0 silk suture and surgically removed leaving the gall bladder intact. This leaves about 30% of the liver mass intact. Forty-eight hours later, the portal vein was cannulated with a 30-gauge needle connected to a model 355 syringe pump (Sage Instruments). About 1 ml of viral supernatant in 8 μg/ml Polybrene was infused over 50 min. To control hemostasis, gel foam was applied to the injection site as the needle was withdrawn at the end of the infusion. Ninety percent of the animals survived both procedures.

Biochemical analysis

The human α1-antitrypsin concentrations were determined by an ELISA that was adapted as described (Armentano et al., 1990) using a human specific antibody that has been used previously in a quantitative radioimmunoassay (Parker et al., 1991). The antibody was conjugated to horseradish peroxidase (Pierce) as described by the manufacturer.
RESULTS

Retroviral mediated gene transfer and expression in hepatocytes in vitro

Three new retroviral vectors were constructed for direct in vivo retroviral gene transfer into the liver (Fig. 1). The LBGPgk vector encodes a fusion β-galactosidase–neomycin phosphotransferase protein that exhibits both enzymatic activities (Friedrich and Soriano, 1991). The LNCMV-hAAT vector (named LNCAAT) has been previously described (Kay et al., 1992). Amphotropic LNCMV-hAAT, LNAAlb-hAAT, GenAlb-hAAT, and LBGPgk-producing cell lines yielded helper-free replication defective viral particles with titers of $1 \times 10^{7}$, $2 \times 10^{7}$, $2 \times 10^{5}$, and $1 \times 10^{6}$ cfu/ml, respectively.

These vectors were used to transduce primary mouse hepatocytes and rat 208F cells. X-gal staining showed that approximately 30–40% of the cells stained blue when infected with the LBGPgk vector (data not shown). Expression of hAAT was determined by measurements of the secreted protein in media after infection. High levels of human AAT was detected in both LNCMV-hAAT and LNAAlb-hAAT infected hepatocytes (Table 1).

<table>
<thead>
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<th>Cell line</th>
<th>LNCMV-hAAT*</th>
<th>LNAAlb-hAAT*</th>
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| Rat 208F embryo fibroblasts were split onto six-well dishes ($2 \times 10^{5}$ cells/dish) 24 hr prior to infection with 2 ml of supernatant from the LNCMV-hAAT or LNAAlb-hAAT amphotropic packaging cell lines for 2 hr. The moi was about 50. Primary C57Bl6 mouse hepatocytes were isolated and plated on 6-cm primaria dishes ($1 \times 10^{5}$ cells/dish) and 48 hr later they infected with the retroviral vectors as described above. The moi was about 100. The supernatants from both cell cultures were removed and the cells were washed twice in PBS. The cells were incubated in fresh media for 24 hr, at which time the media were collected for hAAT determinations. Control determinations were carried out on mouse hepatocytes and 208F cells that were infected with the LBGPgk viral vector. No hAAT was detectable in the controls. All experiments were performed in triplicate. 

In vivo retroviral-mediated gene delivery into mouse hepatocytes

A partial hepatectomy was performed in the animals to stimulate hepatic DNA replication that is required for retroviral integration into the host genome. At various times following the surgical procedure, 1 ml of retroviral supernatant was infused into the portal vein of each animal. When using the LBGPgk vector with a titer of $1 \times 10^{6}$ cfu/ml for hepatic gene transfer, it was possible to estimate the hepatocyte transduction frequency. Hepatocytes were isolated 2 weeks after in vivo viral transduction and the cultured hepatocytes were stained with X-gal (Fig. 2a). Hepatocyte isolation selects against nonparenchymal cells such that over 99% of cells represent bona fide hepatocytes (Kay et al., 1992). To determine further the specificity of transduced cells in the liver, 1 animal was sacrificed 2 weeks after transduction with the LBGPgk vector and frozen liver sections were analyzed by in situ histochemical staining with X-gal (Fig. 2b,c). All of the cells staining blue had characteristic hepatocyte morphology. There was no preferential localization (i.e., perportal) of transduced cells in each section that was examined. Although we cannot rule out the possibility that rare transduced cells in the liver are nonparenchymal, at the very least, most of the transduced cells represent hepatocytes.

The isolated cells were stained 4 hr after plating to avoid counting positive cells that have undergone cell division in culture. The transduction frequency was determined by counting the proportion of blue cells 12 hr after histochemical staining (Fig. 2a). The greatest transduction frequency was 1–2% and was obtained by infusing the virus 48 hr after hepatectomy. These data correlate with maximal DNA synthesis seen in regenerating mouse liver (for review, see Munster, 1975). Amphotropic vectors routinely gave at least 10-fold greater transduction than ectropic vectors (data not shown). We have estimated that approximately $1 \times 10^{7}$ hepatocytes are present in the remaining liver after partial hepatectomy and $1 \times 10^{6}$ viral particles were infused per animal, giving a multiplicity of infection of one viral particle per 10 hepatocytes. With 1–2%
blue hepatocytes, the efficiency of transduction of hepatocytes in vivo under these conditions is about 10–20%.

Several animals were infused with the LBPGpk vector without undergoing prior hepatectomy. Rare blue hepatocytes were detected after X-gal staining of isolated hepatocytes. The transduction frequency in these experiments were approximately 50 to 100-fold lower than with partial hepatectomy.

**Constitutive expression of human α1-antitrypsin in mice after in vivo gene delivery**

To determine whether direct retroviral delivery to hepatocytes in vivo would result in long-term constitutive expression, we developed retroviral vectors that encode the secretable serum hAAT marker. To evaluate for long-term transgene expression serum hAAT levels were measured periodically after transduction with the retroviral vectors. The LNAβ-hAAT vector was transduced into mouse hepatocytes. One animal had steady-state serum hAAT levels of over 1 μg/ml (Fig. 3A). Although variability was seen between animals, the most important finding was the constitutive nature of transgene expression that was observed for 6 months in individual animals.

Similar experiments were performed with the GenAlb-hAAT vector. In these animals constitutive, albeit low-level, expression was achieved for at least 6 months (Fig. 3b). The discrepancy in expression from the LNAβ-hAAT and GenAlb-hAAT vectors, which both utilize the albumin promoter—enhancer to drive hAAT expression, is most likely the result of a 50- to 100-fold difference in viral titers and thus a lower transduction frequency in vivo.

When the LNCMV-hAAT vector was transduced into hepatocytes in vivo, a large peak of serum hAAT was seen during the first 1½ weeks (Fig. 3C), but the level fell quickly. Similar results were obtained in a previous study when canine hepatocytes were transduced with this vector and autologously transplanted back into the animal (Kay et al., 1992). This is consistent with transient gene expression from the cytomegalovirus (CMV) promoter in hepatocytes in vivo. There is a low level of constitutive expression from some of the mice in which the transduced cell type is not known at this time.

Some animals underwent infusion of the LNCMV-hAAT or LNAβ-hAAT viral supernatants without undergoing a partial hepatectomy. The expression from the LNAβ-hAAT transduced mice was about 30 to 100-fold lower than the hepatotomized animals. This is in good agreement with animals that were transduced with the LBPGpk vector without prior hepatectomy. Additionally, animals were injected with heat-denatured retrovirus after partial hepatectomy. This treatment did not affect the quantity of hAAT in the viral supernatants that was infused into the animals. Serum hAAT measured in these animals was negligible (less than 6 ng/ml) and transient for each vector tested (data not shown).

**DISCUSSION**

In this study, we demonstrate that retroviral vectors can be used efficiently to transduce hepatocytes in vivo by a relatively simple method that results in long-term constitutive gene expression in individual recipient animals. Kaeko et al. (1991) have used carbon tetrachloride to induce liver regeneration followed by direct liver infusion of a retroviral vector in the mouse that contains the neomycin phosphotransferase gene. By polymerase chain reaction (PCR) analysis, they were able to demonstrate a transduction frequency of about 1/100 liver cells. This study did not conclusively demonstrate which cell types in the liver were genetically modified. Ferry et al. (1991) recently reported the transduction of rat hepatocytes in vivo with an E. coli β-galactosidase containing retroviral vector. This method utilizes a complex in situ liver perfusion technique 24 hr after partial hepatectomy. Although β-galactosidase activity was detected in hepatocytes at 6 months after transduction, only single time points were analyzed in each animal, and it is not possible to study the level of transgene expression over time in individual recipients animals. In this study, by using a serum-secretable marker in combination with the intracelarular β-galactosidase marker it was possible to demonstrate parenchymal cell specificity and long-term constitutive expression.

Because in vitro gene expression does not necessarily correlate well with in vivo gene expression (Clayton et al., 1985; Isom et al., 1987), this relatively simple method will allow testing of a large number of recombinant retroviral vectors in vivo. The CMV promoter—enhancer has been used to express genes in many cells types and has been shown to be transcriptionally active in vitro but most recently has been demonstrated to be inactivated in a number of tissues in vivo (Schmidt et al., 1990; Scharfman et al., 1991; Kay et al., 1992), including the liver (Schmidt et al., 1990; Kay et al., 1992). Results from this study support this finding because peak serum hAAT levels are seen transiently in mice that have been transduced with the LNCMV-hAAT vector. The methods we have developed offer a valuable model for studying the expression of exogenous genes delivered to hepatocytes in vivo. This will be particularly important when designing vectors for the therapeutic treatment of genetic disorders through gene replacement therapy.

An extensive effort was placed into developing an ex vivo approach for hepatic gene therapy, which is technically complex. Although this approach has been conceptually important.

![Fig. 2.](image-url)
to illustrate the feasibility of somatic gene therapy, the procedure is extremely complicated in its entirety and the potential for its wide-scale application in clinical medicine is rather limited. The current method of retroviral vector delivery of therapeutic genes to hepatocytes in vivo is a simple procedure applicable to a variety of hepatic disorders.

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