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Hepatic Gene Therapy: Efficient Retroviral-Mediated Gene Transfer into Rat Hepatocytes In Vivo

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Abstract—*The rat is an excellent model for gene therapy because there are many rat models for human diseases. We have developed a simple and efficient method to deliver genes to the rat liver using recombinant retroviral vectors. A 70% partial hepatectomy followed by retroviral infusion into the portal vein results in 10–15% hepatocyte transduction in vivo. This is 10 times more efficient than in the mouse due partially to the observation that the rat livers have much more synchronous hepatocyte replication after partial hepatectomy. Using a recombinant retroviral vector containing the human α_1-antitrypsin cDNA, persistent expression of the human protein in recipient rat plasma was observed for at least six months and at a level that is 10 times greater than the mouse. Thus, rats can serve as an excellent model for gene therapy of metabolic disorders secondary to hepatic deficiencies.*

INTRODUCTION

The rat is an extensively investigated animal, and there are many excellent rat models of human genetic and epigenetic diseases (1). In order to investigate the feasibility of treating some of these disease models using the techniques of somatic cell gene transfer, we wanted to develop simple and efficient methods for gene therapy in the rat. An excellent target organ for gene therapy is the liver because it is a large, metabolically active organ, and many genetic diseases result from mutations in genes expressed in the liver (2). Hepatocytes also have a slow turnover rate (3), and thus genetically modified cells will persist long-term.

Presently, the best vector for stable introduction of genes into somatic cells in animals is the recombinant, replication-defective retrovirus. The retrovirus is attractive because it integrates into the host genome and is thus permanent. The problem with using retroviruses to transduce hepatocytes is that retroviruses require host cell division for efficient integration into the genome (5). However, in a normal, healthy liver, very few hepatocytes are replicating (3). To stimulate hepatocyte replication, several laboratories have reported performing a 70% partial hepatectomy on rats. When the remaining hepatocytes divide to regenerate the liver, they are susceptible to retroviral transduction. The retrovirus is delivered by vascular isolation of the liver, followed by perfusion of the liver with retroviral supernatant (6, 7). Although this is an efficient method for gene delivery (5–20% of the hepatocytes were transduced), it is complicated by the need for extensive and elaborate surgery.

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Recently, Kay et al. (8) performed a 70% partial hepatectomy in mice followed by infusion of retrovirus into the portal vein without vascular isolation. Using a retrovirus encoding the β-galactosidase gene, this technique resulted in 1–2% hepatocyte transduction as determined by X-gal staining. They also introduced the gene for the secreted reporter protein human α_1-antitrypsin (hAAT) and detected constitutive levels of hAAT in mouse serum for more than 200 days. This method, applied in the rat, resulted in 10–15% hepatocyte transduction and constitutive expression of the human protein at a level 10-fold greater than in the mouse.

MATERIALS AND METHODS

Animals and Partial Hepatectomy. Male Lewis rats, 3–4 weeks old were purchased from Harlan, Sprague Dawley Inc. The animals were housed in a vivarium with a 12-h light–dark cycle with water and food (standard laboratory chow) provided ab libitum. The partial hepatectomy was performed under a general combination anesthetic: ketamine (42.8 mg/ml), xylazine (8.6 mg/ml), and acepromazine (1.4 mg/ml), administered at 0.5–0.7 ml/kg. The 70% partial hepatectomy involved removal of the median and left lateral lobes and was performed according to Higgens and Anderson (9). The skin was then closed with autoclip 9-mm wound clips.

Retrovirus Harvest and Preparation. The virus-producing cells were cultured at 37°C with 5.0% CO_2 in 150-mm tissue culture plates with 25 ml of media (high glucose D-modified Eagle's media supplemented with 10% Hyclone bovine calf serum and 1 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycine). When the cells were 70–80% confluent, the medium was replaced with 15 ml of fresh medium; 18 h later, the medium was harvested, filtered through a 0.45-μm syringe filter, and polybrene was added to 8.0 μg/ml. The retroviral medium was infused into animals within 1 h of collection.

Infusion of Retrovirus into Remaining Lobes of Liver. At indicated times after the 70% partial hepatectomy, rats were anesthetized as above and opened through the same incision used for the partial hepatectomy. The portal vein was cannulated with a 24-G catheter connected to a 10-cc syringe by a 30-in. extension set. Over the course of 20–30 min, 3.0 ml of retroviral supernatant was infused using a Sage Instruments syringe pump (model 355). After infusion, the catheter was removed and pressure was applied for 10–30 min to control bleeding. The abdominal muscle was sutured with Chromic Gut and the skin closed with autoclips.

Isolation and X-Gal Staining of Hepatocytes. The technique for hepatocyte isolation is adapted from Berry and Friend (10). Briefly, 10 days after retroviral infusion, the rat was anesthetized, the portal vein was cannulated with a 20-G catheter, and the inferior venae cava cut. The liver was then perfused with 150 ml of Earle's balanced salt solution without calcium or magnesium (EBS^-) plus 0.5 mM EGTA, 50 ml of EBS^-, and finally 150 ml of Earle's buffered solution with calcium, with 0.3 mg/ml Boehringer collagenase and 0.05 mg/ml Sigma soybean trypsin inhibitor. All the above solutions were warmed to 37°C and infused at 20 ml/min. Hepatocyte culture conditions and media were described previously (11). The hepatocytes were cultured for 16–20 h before X-gal staining, performed as described previously. Histological X-gal staining of frozen liver sections was performed seven days after retroviral infusion and was described previously (11).

Generation of LX/hAAT Retrovirus. LX/hAAT was constructed by removing the *neo* gene and CMV promoter from LNCX by a BclI (blunted), HindIII restriction enzyme digest, and inserting the hAAT coding region

as a SmaI, HindIII fragment. The LX/hAAT construct was then electroporated into GPAM-12 packaging cells, and individual virus producing colonies were selected and screened for retroviral production (retroviral supernatant from individual colonies were used to transduce rat embryo fibroblast cell line 208F and hAAT production from the fibroblasts was assayed). The apparent titer of LX/hAAT is at least 2×10^6 PFU/ml as determined by comparing the amounts of viral RNA in the supernatant from LX/hAAT producing cells to a retrovirus of known titer (data not shown).

Detection of hAAT in Rat Serum. Rat serum was isolated and the concentration of human α_1-antitrypsin was determined by an ELISA as described in Kay et al. (8).

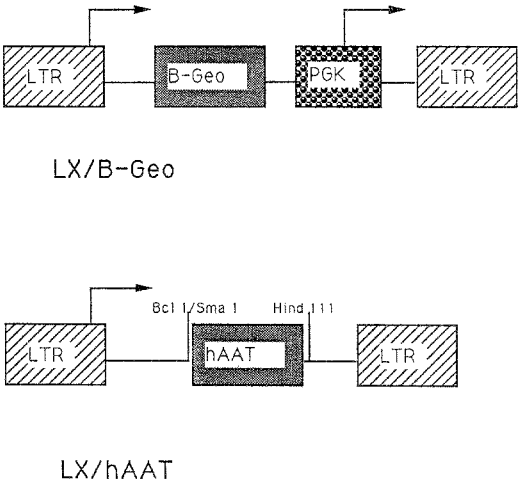

Fig. 1. Diagrams of the retroviruses used in this study. (A) LX/β-Geo, β-Geo is a fusion gene of the neomycin phosphotransferase gene and the β-galactosidase gene. β-Geo is under the transcriptional control of the Moloney murine leukemia virus (MMLV) long terminal repeat (LTR). The retrovirus also encodes for the phosphoglycerol kinase promoter (PGK). (B) LX/hAAT encodes the human α_1-antitrypsin gene under the transcriptional control of MMLV LTR. The arrows indicate transcriptional start sites.

RESULTS

Recombinant Retroviral Vectors. The amphotropic retrovirus LX/β-Geo (Fig. 1A) has been described previously (8). Briefly, β-Geo is a fusion of the *E. coli* β-galactosidase gene and the neomycin phosphotransferase gene. The fusion protein retains both enzymatic activities. The β-Geo gene is under the transcriptional control of the Moloney murine leukemia virus (MMLV) long terminal repeat (LTR) described by Miller and Rosman (12). The retrovirus is produced from the amphotropic retroviral packaging cell line GPAM-12 and has a titer of 1×10^6 PFU/ml. The LX/hAAT retrovirus (Fig. 1B) encodes the human α_1-antitrypsin gene (8) under the transcriptional control of the MMLV-LTR (12).

In Vivo Retroviral Transduction of Rat Hepatocytes. The MMLV vectors require a cell to be dividing in order to integrate into the host genome. To stimulate hepatocyte replication, a 70% partial hepatectomy can be performed. Since young rats (3–4 weeks old) have a greater rate of DNA synthesis than older rats after partial hepatectomy (13), their hepatocytes will be more susceptible to retroviral transduction. A 70% partial hepatectomy was thus performed on 3 to 4-week-old male Lewis rats and 24 h later, 3 ml of the β-Geo retrovirus was infused into the portal vein over the course of 30 min. Seven days later, the liver was isolated and the frozen section was stained for X-gal. As can be seen in Fig. 2A, there are many cells that have stained in the liver infused with the β-Geo retrovirus, while no blue cells are visible in liver after only a partial hepatectomy and mock infusion (Fig. 2B). It is known that the amphotropic retrovirus can transduce vascular endothelial cells in vivo (4). However, of the sections we inspected, transduction was limited to cells with hepatocyte morphology (Fig. 2A). The blue cells are relatively evenly dispersed in the liver parenchyma, although some appeared in groups and rows, perhaps indicating limited division of hepatocytes following transduction.

Time Course for Optimal Retroviral Transduction of Rat Hepatocytes In Vivo. To deter-

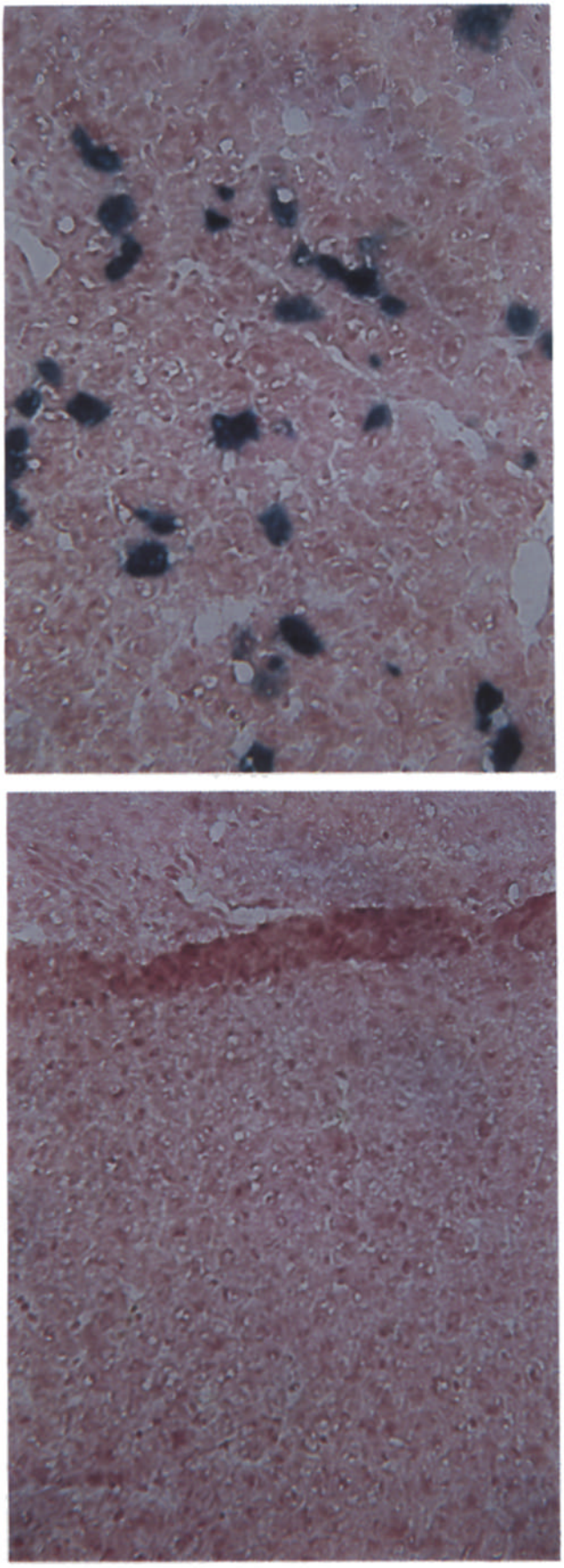

Fig. 2. In vivo transduction of rat hepatocytes with LX/β-Geo retrovirus. A 70% partial hepatectomy was performed on 3 to 4-week-old rats; 24 h later, either (A) 3.0 ml of LX/β-Geo retrovirus or (B) 3.0 ml of medium was infused into the portal vein. Seven days later, the liver was isolated, frozen, sectioned, and stained with X-gal and counterstained with nuclear fast red. Pictures represent sections from one of three rats infused with β-Geo, and one of one mock infused. Blue cells were only detected in the liver sections infused with LX/β-Geo retrovirus.

mine when to infuse retrovirus after partial hepatectomy for optimal transduction of rat hepatocytes, retrovirus was infused into rats at 0, 12, 18, 24, and 36 h after partial hepatectomy. In order to better quantitate the transduction efficiency and to ensure the blue cells were hepatocytes, we isolated and cultured the hepatocytes under conditions that selected for the growth of hepatocytes. Ten days after retroviral infusion, hepatocytes were isolated by collagenase perfusion and cultured overnight as described in Materials and Methods. Even if cell division occurs, the percentage of blue cells should not change, unless transduction affects hepatocyte growth. Twelve hours after X-gal staining, the percent transduction was calculated by scanning random fields at 400× magnification and counting the number of blue cells and the total number of cells (at least 700). The optimal time for retroviral infusion appears to be 24 h after partial hepatectomy, at which time, 10–15% of the hepatocytes were transduced (Fig. 3).

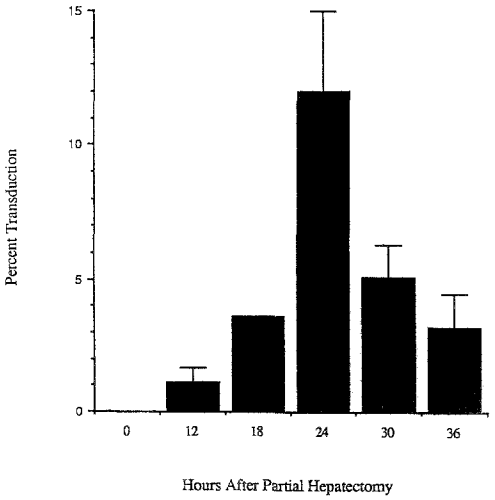

Fig. 3. Time course for in vivo infection of rat hepatocytes. The 70% partial hepatectomies were performed on 3 to 4-week-old male Lewis rats. Then at time 0 h ($N = 2$), 12 h ($N = 5$), 18 h ($N = 1$), 24 h ($N = 5$), 30 h ($N = 4$) or 36 h ($N = 3$) after partial hepatectomy, 3.0 ml of retroviral supernatant was infused into the portal vein. Seven to 10 days after transduction, the hepatocytes were isolated, cultured for 18 h, and stained with X-gal. Plates of hepatocytes were scanned at 400× magnification and random fields were counted for the number of blue cells and the total number of cells. At least 700 cells were counted per time point. Bars represent mean and standard deviation. Range at 24 h is 8.6–16.7%.

Retroviral Transduction Efficiency of Rat and Mouse Hepatocytes In Vitro. Kay et al. (8) reported that using this in vivo retroviral transduction method in the mouse, they achieved a hepatocyte transduction efficiency of 1–2%, while we observed a 10–15% transduction efficiency in the rat. The 10-fold difference in transduction efficiency is not due to inherent differences in susceptibilities to infection by amphotropic retrovirus, since rat and mouse hepatocytes are transduced 20–25% with an amphotropic retrovirus in culture (14, 15, and Dr. M. Kay, personal communication). Thus both the rat and mouse hepatocytes are equally susceptible to amphotropic retroviral transduction.

Histological Study of Regenerating Liver of Rat and Mouse. Since both rat and mouse hepatocytes are equally susceptible to retroviral transduction in vitro, we investigated the regenerating livers of rats and mice. Perhaps, if the rat has a more vigorous hepatocyte replication response after partial hepatectomy than the mouse, it would explain the difference in transduction efficiency. The 70% partial hepatectomies were performed on rats and mice, and the remaining liver lobes were isolated at times when in vivo retroviral transduction is known to be optimal (24 h for the rat and 48 h for the mouse). Random fields were scanned at 400× magnification and the number of mitotic figures and the total number of cells was determined. The mitotic index is the number of mitotic figures per 1000 cells (3). The rat liver had a mitotic index of 84.8, while the mouse liver had a mitotic index of 24.6. In both animals, the mitotic figures did not appear localized in any region of the liver (i.e., periportal).

Expression of Human α_1-Antitrypsin in Transduced Rat Hepatocytes. In order to study the expression of retrovirally transduced

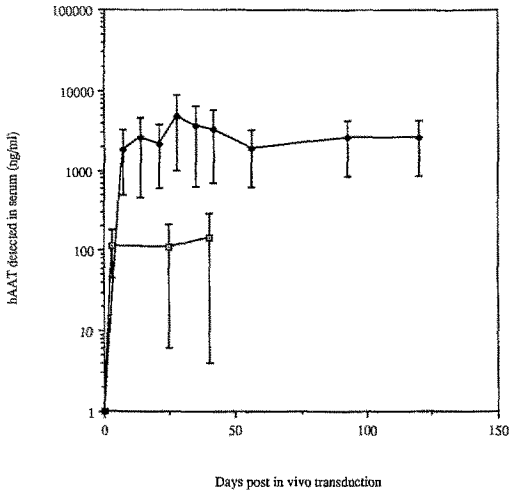

Fig. 4. Detection of human α_1-antitrypsin in the serum of rats (closed squares) and mice (open squares) after in vivo transduction of hepatocytes with LX/hAAT retrovirus. The 70% partial hepatectomies were performed on mice and rats. The rats were infused 24 h later with 3.0 ml of supernatant containing the LX/hAAT retrovirus, while the mice were infused at 48 h with 1.0 ml of supernatant. Serum samples were collected from the animals at the indicated times and assayed for hAAT. The graph represents the mean ± standard deviation for $N = 4$ (mouse) and $N = 7$ (rat).

genes long-term, we transduced rat hepatocytes with a retrovirus encoding the gene for human α_1-antitrypsin (hAAT). hAAT was chosen because, being a secreted protein, its presence can be detected easily in the serum of the animal, and expression can be monitored long-term in each animal. hAAT has also been shown not to elicit an immunological response in dogs and mice when expressed from hepatocytes, but it can be detected and quantitated by an ELISA assay. A 70% partial hepatectomy was performed on rats, followed 24 h later by infusion of 3 ml of supernatant containing the LX/hAAT retrovirus. As can be seen in Fig. 4, hAAT can be detected in the rat serum seven days after retroviral transduction. The average levels reached 2000 ng/ml within a week after viral infusion and remained steady for at least 120 days. No hAAT was ever detected in the serum of rats transduced with a control retroviral vector (data not shown).

Mouse hepatocytes were also transduced in vivo with the same retrovirus under optimal conditions, and the presence of hAAT was monitored in their serum. hAAT levels in the mouse have also remained steady for 40 days of monitoring. As can be seen in Fig. 4, the average level of hAAT in the mouse of about 100–200 ng/ml is 10-fold lower that in the rat. This observation is in agreement with the results from the β-gal staining showing that hepatocyte transduction in the rat is 10-fold higher than in the mouse.

DISCUSSION

A 70% partial hepatectomy followed by infusion of retrovirus into the portal vein results in 1–2% hepatocyte transduction in the mouse (8), while in the rat, the same technique achieves 10–15% hepatocyte transduction. This difference is not due to differences in susceptibilities to retroviral transduction since both rat and mouse hepatocytes are equally transduced by amphotropic retrovirus in vitro. The difference is also not due to a greater number of virus particles being infused into the rat since we infused 3.0 ml into a 50-g rat (or 6×10^4 PFU/g body weight) while Kay et al. (8) infused 8×10^4 PFU/g into the mouse. The difference may be due, in part, to the fact that at the time for optimum transduction by retrovirus, the rat liver has a three to fourfold higher mitotic index than the mouse.

The percentage of transduced cells by our method (10–15%) is comparable to that achieved using the vascular isolation method (5–20%) of Ferry et al. (6) and 16% by Rozga et al. (7). A critical difference is that while the other groups' methods require very intricate surgical procedures to vascularly isolate the liver before infusion of retrovirus into the portal vein, our method only requires infusion of the retrovirus into the portal vein.

To study the expression of transduced

genes long-term, we used the secreted marker protein human α_1-antitrypsin (hAAT). Using hAAT as a marker, one can study both the level of expression and changes in expression levels in one animal. This is superior to transducing several animals and sacrificing them at different times to determine percentage of cells still expressing the gene of interest, since one then introduces variability between animals. The amounts of hAAT being expressed varied from animal to animal. The levels ranged from 200 ng/ml to 4000 ng/ml. The levels of hAAT appear to be steady for at least six months. With respect to hAAT production, the mice produced up to 100–200 ng/ml of hAAT, while the rats averaged 2000 ng/ml. This is the expected result since 10-fold more hepatocytes are shown to be transduced by X-gal staining in the rat than in the mouse.

The focus of this study was to develop a simple and efficient method to deliver genes to the rat liver using recombinant retroviral vectors. We determined that a 70% partial hepatectomy followed 24 h later by infusion of 3 ml of retrovirus into the portal vein results in 10–15% hepatocyte transduction. Genes introduced in this manner are also expressed for at least six months. These results suggest that the rat is an excellent model for hepatic gene therapy of metabolic disorders.

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