Expression of human α1-antitrypsin in dogs after autologous transplantation of retroviral transduced hepatocytes

gene therapy/canine hepatocyte/autologous transplantation/cytomegalovirus promoter

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ABSTRACT  The liver represents an excellent organ for gene therapy since many genetic disorders result from the deficiency of liver-specific gene products. We have previously demonstrated that transgenic mouse hepatocytes can be heterologously transplanted into congenic recipients where they survived indefinitely and continued to function as hepatocytes. Here we demonstrate the autologous transplantation of retrovirally transduced canine hepatocytes. At least 1 x 10^9 hepatocytes or 5% of the liver mass can be transplanted by the portal vasculature. In two animals we have transplanted hepatocytes transduced with a retroviral vector containing the human α1-antitrypsin cDNA under transcriptional control of the cytomegalovirus promoter. Both animals had significant human α1-antitrypsin in the serum for 1 month. Although the serum levels of human α1-antitrypsin eventually fell due to inactivation of the cytomegalovirus promoter, PCR analysis demonstrated that a significant fraction of transduced hepatocytes migrated to the liver and continued to survive in vivo. The results suggest that gene therapy of hepatic deficiencies may be achieved by hepatocellular transplantation after genetic reconstitution with the use of promoters of cellular genes that are active in the normal liver.

Many laboratories have developed methods for the transduction of genes into cultured primary hepatocytes (1–5). To use this method for gene therapy, one must be able to introduce the genetically altered cells back into the animal, preferably to the liver. Using transgenic mouse hepatocytes as a model, we (6) and others (7) have recently demonstrated that freshly isolated mouse hepatocytes can be transplanted into the liver of congenic recipients by direct splenic or portal vein injection. The grafted cells migrated to the liver and functioned indefinitely as hepatocytes after transplantation.

The next critical step is to determine whether hepatocytes that undergo in vitro manipulation and retroviral transduction can be transplanted back into the animal and function in a manner similar to the transgenic mouse models. Wilson et al. (8) transplanted heterologous hepatocytes after retroviral transduction with the human low density lipoprotein receptor into low density lipoprotein-deficient rabbits but found limited short-term expression. We have, however, selected the canine model to develop methodologies for autologous transplantation of virally transduced hepatocytes because (i) the size of a young dog parallels that of a human infant and (ii) there are canine hepatic-deficiency disorders that can be used as models for gene therapy. We selected the human α1-antitrypsin (hαAT) cDNA as a marker gene since it encodes a secretable protein that can be easily detected in the serum of recipients with an RIA that utilizes a species-specific hαAT antibody. We show here that at least 1 x 10^9 hepatocytes can be transplanted into the animals and, after retroviral gene transduction, autologous hepatocytes transplanted by the portal circulation remain in the liver.

MATERIALS AND METHODS

Animals. Beagles were purchased from Ridgian Farms (Mount Horeb, WI) and were cared for and used in accordance with the National Institutes of Health guidelines as dictated by the Baylor animal care facility. All animals used were 8–16 weeks old.

Surgical Procedures. All operative procedures were performed under general anesthesia. A midline abdominal incision was made and the left lateral lobe of the liver was isolated and removed. In some animals, at the time of partial hepatectomy, a 5 French Hydrocath catheter (Norfolk Medical, Skokie, IL) was cannulated into a mesenteric or splenic vein. A subcutaneous injection port connected to the catheter was placed over the right lateral portion of the abdominal wall.

Hepatocyte Isolation. The isolated liver lobe was immediately cannulated with a 16-gauge catheter and manually perfused with 200–300 ml of magnesium/calcium-free Earle’s balanced salt solution (GIBCO) containing 0.5 mM EDTA (solution 1). The catheter was connected to a pump and the lobe was perfused with solution 1 at 80–100 ml/min for an additional 3–5 min. Without interruption, solution 2 containing Earle’s balanced salt solution (with calcium and magnesium), collagenase B (lot CKA 139, Boehringer Mannheim) at 0.4 mg/ml, and soybean trypsin inhibitor (Sigma) at 0.05 mg/ml was perfused at a rate of 65–100 ml/min for 12–20 min. The cells were isolated in solution 3 containing basal medium [minimal essential medium/Waymouth, 3:1 (vol/vol), both without tyrosine], 10% (vol/vol) fetal calf serum, 10 mM Hepes (pH 7.4), penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM glutamine, 1 μM dexamethasone, and insulin (10 μg/ml). The cells were plated on Primaria culture dishes (Falcon) in solution 3 as described (5). Four hours later, the medium was changed to a serum-free medium containing solution 3 without fetal calf serum but supplemented with glucagon (1 μg/ml), epidermal growth factor (50 ng/ml), prolactin (20 milliunits/ml), human growth hormone (10 microunits/ml), linoleic acid (5 μg/ml), 30 nM selenium, transferrin (10 μg/ml), 1 μM 3,5,3′-triiodothyronine, nones-

Abbreviations: hαAT, human α1-antitrypsin; Neo, neomycin phospho- transferase; CMV, cytomegalovirus.
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sential amino acid mixture (0.1 mM, Gibco), and Gly-His-Lys (20 ng/ml).

**Retroviral Transduction.** Retroviral transduction of hepatocytes was as described (5) except the initial Polynene concentration was 4 μg/ml. The multiplicity of infection was 10.

**Transplantation of Hepatocytes.** The cultured hepatocytes were treated with trypsin by rinsing the plates with 0.05% trypsin/0.53 mM EDTA (Gibco) and incubating at 37°C for 5–15 min. The cells were rinsed off the plates in 2–10% serum containing medium (either dog or fetal calf serum) and washed two to five times. In some cases the cells were filtered over a nylon mesh before resuspension in basal medium containing 10 mM Hapes and 1–5% (vol/vol) allogeneic dog serum. Hepatocytes transplanted immediately after collagenase isolation were rinsed in a similar manner and resuspended in the same basal medium containing dog serum. Transplants 1–4 were performed manually with a syringe over 30 min. Direct splenic injections were performed with a 20- or 22-gauge needle. Later transplants were performed with an infusion pump at a rate of 0.5–2 ml/min by the subcutaneous injection port. Except where noted, hepatocellular transplants were performed 2.5–6 days after *in vitro* culture. The concentration of cells varied between 1 × 10⁶ and 1 × 10⁷ cells per ml.

**Retroviral Vectors.** The LNCX retroviral vector (9) was kindly supplied by Dusty Miller (Fred Hutchinson Cancer Research Center, Seattle). The EcoRI fragment encoding the human hAAT cDNA (10) was blunt-ended with the Klenow fragment of DNA polymerase I and then ligated into the Hpa I site in LNCX. The LNCAT DNA was transfected into the GP+5-E-86 (11) packaging cell line and high-titer clones (2 × 10⁶ colony-forming units/ml) were isolated by G418 selection. These clones did not produce wild-type virus (12). Filtered supernatants from the ecotropic packaging lines were used to infect the amphotropic GP+AM12 (13) packaging cell lines. High-titer clones (1 × 10⁷ colony-forming units/ml) were isolated in a similar fashion and used to infect canine hepatocytes. The packaging lines were kindly provided by Arthur Bank (Columbia University, New York).

**Genomic DNA Isolation and PCR Analysis.** DNA was isolated from sections of liver or cultured cells as described (13) except that the DNA was phenol/chloroform extracted, ethanol-precipitated, and resuspended in water. PCR amplification using Neo-1 and Neo-2 primers was as described (14).

**RESULTS**

**Canine Hepatocyte Isolation and Transplantation.** The initial experiments were aimed at developing methods for scaling up hepatocyte isolation and at transplanting autologous hepatocytes after *in vitro* gene transduction. Table I summarizes the first 10 hepatocellular transplants performed by various methods. Seven dogs had partial hepatectomy and autologous cell infusions after *in vitro* culture. In transplants 1–3, there were technical difficulties with canine hepatocyte preparations, culture conditions, and cell retrieval by trypsin treatment. After optimization of procedures, 1–3 × 10⁹ hepatocytes were consistently obtained from a single lobe. Cell viability at isolation and after trypsin treatment was 70–95% as estimated by cell plating in culture and by trypan blue exclusion.

In experiments 4–7, hepatocytes were transplanted by direct splenic injection or by the portal vasculature. The animals developed shock symptoms that included lethargy, pallor, hypotension, and hematemesis. Most of the episodes were transient and resolved within minutes after the infusion was stopped. Two of the animals died and necropsy revealed serosanguinous ascites, small bowel hemorrhage, and spleen and liver infarction. The liver vasculature and portal vein were engorged and thrombosed with hepatocytes. In the two surviving animals, there were no associated long-term effects. These animals were sacrificed with unremarkable findings at necropsy.

Three additional heterologous transplants were performed with freshly isolated hepatocytes by three methods while monitoring the portal vein and femoral artery pressures in each animal during transplantation. An increase in portal vein pressure prior to a systemic drop in blood pressure would suggest a mechanical obstruction in the portal vasculature. However, no difficulties were associated with the transplantation of 1 × 10⁹ hepatocytes into each animal (Table I, animals 8–10). Liver and spleen biopsies were obtained at the end of the transplantation period and histologic examination of the liver identified hepatocytes in the portal vasculature (data not shown). After the transplantation, to replicate the shock conditions, components of the medium were infused and, after infusion of fetal calf serum in dog 9, the mean arterial pressures dropped from ≈80 mmHg to ≈35 mmHg for several hours. Serum infusions into normal dogs also elicited a transient clinical shock response as mentioned above. This suggests that the shock episodes were vasoactive in origin and prompted more extensive washing of hepatocytes prior to the hepatic transplants.

**Transduction of Hepatocytes with a Recombinant Retroviral Vector.** A retroviral vector encoding the hAAT cDNA was constructed (Fig. 1A) and the conditions leading to the greatest transduction efficiency of canine hepatocytes were determined. The optimal time of infection was 32–36 h after isolation and the optimal density of plating was 2 × 10⁶ cells per 100-mm culture dish. Under these conditions, ≈25% of the cells stained with 5-bromo-4-chloro-3-indoly  β-galactoside when a β-galactosidase-containing retroviral vector (5) was used for transduction (data not shown). These

Table 1. Transplantation of hepatocytes in dogs

<table>
<thead>
<tr>
<th>Animal</th>
<th>Body weight, kg</th>
<th>Number of hepatocytes isolated</th>
<th>Number of hepatocytes transplanted</th>
<th>Transplant route</th>
<th>Animal response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.5</td>
<td>1.2 × 10⁹*</td>
<td>4 × 10⁷</td>
<td>Spleen</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
<td>1.5 × 10⁹*</td>
<td>2 × 10⁸</td>
<td>Spleen</td>
<td>Normal</td>
</tr>
<tr>
<td>3</td>
<td>6.5</td>
<td>1.9 × 10⁹</td>
<td>4 × 10⁸</td>
<td>Spleen (2 sites)</td>
<td>Normal</td>
</tr>
<tr>
<td>4</td>
<td>3.4</td>
<td>3.0 × 10⁹*</td>
<td>7.5 × 10⁸</td>
<td>Spleen (4 sites)</td>
<td>Died 2 h after transplant</td>
</tr>
<tr>
<td>5</td>
<td>7.0</td>
<td>2.4 × 10⁹</td>
<td>1 × 10⁸</td>
<td>Mesenteric vein (cath)</td>
<td>Shock and recovery</td>
</tr>
<tr>
<td>6</td>
<td>4.0</td>
<td>3.5 × 10⁹*</td>
<td>9 × 10⁸</td>
<td>Splenic vein (cath)</td>
<td>Multiple shock and recovery</td>
</tr>
<tr>
<td>7</td>
<td>5.0</td>
<td>3.0 × 10⁹*</td>
<td>2.5 × 10⁸</td>
<td>Splenic vein (cath)</td>
<td>Shock and death</td>
</tr>
<tr>
<td>8</td>
<td>4.7</td>
<td>1.5 × 10⁹</td>
<td>1 × 10⁹</td>
<td>Spleen</td>
<td>Normal</td>
</tr>
<tr>
<td>9</td>
<td>6.7</td>
<td>1.5 × 10⁹</td>
<td>1 × 10⁹</td>
<td>Splenic vein (cath)</td>
<td>Normal</td>
</tr>
<tr>
<td>10</td>
<td>4.5</td>
<td>1.3 × 10⁹</td>
<td>1 × 10⁹</td>
<td>Splenic artery (cath)</td>
<td>Normal</td>
</tr>
</tbody>
</table>

*cath, Catheter.*

*Retroviral-infected hepatocytes.
plating and infection conditions were used in subsequent experiments. For transplantation experiments, $2 \times 10^9$ hepatocytes were cultured on 1000 plates. Approximately 4–5 liters of fresh viral supernatant was prepared for each experiment. The culture conditions used selected against nonhepatocyte liver cells (15) and, by both morphology and staining with antibody to a hepatocyte-specific enzyme marker, glutamine synthetase (16), most of the cells (≈99%) are hepatocytes (data not shown). Six days after infection or mock-infection with the LNCAAT retroviral vector, the hepatocytes were stained with an antibody specific for hAAT. Twenty to 25% of the transduced cells were positive for hAAT with variable intensity (Fig. 1B), whereas none of the mock-infected cells was stained with this antibody (Fig. 1C).

**Autologous Hepatocyte Transplantation After Retroviral Transduction.** In two animals (A1 and A2), hepatocytes that were transduced with the LNCAAT retroviral vector were autologously transplanted by the splenic vein. Fig. 2 shows the *in vitro* production of hAAT in hepatocytes after viral transduction. The levels reached 50–75 μg per $2 \times 10^6$ plated cells per 48 h at 4–6 days after retroviral infection. After trypsin treatment, trypan blue exclusion revealed 97% and 75% hepatocyte viability in A1 and A2, respectively. Trypsin treatment and replating did not have a significant effect on hAAT production in transduced hepatocytes (Fig. 2).

Transplantation of hepatocytes was started 12 h after retroviral infection; cells were injected three times over a 24-h period for A1 or twice over a 12-h period for A2. During the transplantation procedures, the two animals suffered several minor shock episodes as described above. The *in vivo* secretion of hAAT was determined by periodic measurements of serum hAAT in the transplanted animals as shown in Fig. 3. The peak hAAT concentration was 4.6 μg/ml in animal A1 and 1.2 μg/ml in animal A2 obtained 6–8 days after
transplantation. The greater expression of hAAT in A1 correlates with a greater proportion of viable hepatocytes that were transplanted. The hAAT concentration began to fall at day 10 and reached undetectable levels after day 47 in A1 and day 35 in A2.

Long-Term Survival of Transduced Hepatocytes in the Recipient Liver. There are several possible explanations for the loss of hAAT production in the serum after transplantation. The first possibility is that the animal makes antibodies to hAAT leading to a fall in serum levels. This was not the case in A1 as the serum did not contain antibodies to hAAT when assayed (data not shown). The other possibilities are (i) the cultured hepatocytes may survive for only a short period after transplantation and (ii) the hAAT transgene is turned off in vivo. To determine whether the transduced cells were still present after serum hAAT levels became undetectable, 1.5 and 4.5 months after transplantation, animal A1 underwent open liver biopsy. Multiple liver wedges were obtained from several portions of each lobe. In addition, hepatocytes were prepared from one lobe and cultured in the usual manner. Genomic DNA was isolated from hepatocytes after 1 week of culture and from samples obtained from different liver lobes. Positive control DNA was from nontransplanted hepatocytes that were transduced with the LNCAAT retroviral vector. These genomic DNAs were subjected to the PCR using a set of Neo-specific primers. To estimate the proportion of LNCAAT transduced cells present in the liver, the positive control DNA was serially diluted by factors of 10 with uninfected canine DNA. The PCR product is a 320-nucleotide fragment that could be detected in material from all liver tissues examined by ethidium bromide staining except for the uninfected canine DNA used as a negative control (Fig. 4A, lanes a, f–m). The gel was then blotted and probed with a radioactive Neo probe. The same specific 320-base-pair fragment hybridized with the radioactive probe (Fig. 4B, lanes b–e and f–m), proving specificity of the PCR amplification product. The linear autoradiograph signal of the positive control DNA was from dilutions 1:100 to 1:10,000 (Fig. 4B, lanes c–e). Thus, the DNA isolated from transplanted hepatocytes was diluted 1:10 prior to amplification so that the autoradiograph signals were in this linear range (Fig. 4B, lanes g, i, and k). The intensities of the diluted samples were approximately the same as the 1:1000 diluted positive control DNA, suggesting that ≥1 in 400 hepatocytes present in A1 after transplantation was transduced with the LNCAAT retroviral vector.

The total number of viable cells transplanted in animal A1 was 3.8 × 10^9. Hepatocytes transplanted in the mouse have 20–95% long-term survivability (6). If the liver mass of the animal is 3.5% of the total body weight and there are 1 × 10^8 hepatocytes per g of tissue, then the total number of hepatocytes is 1.8 × 10^10. If the survival is 50%, then the transplanted cells would represent ≥1% of the original total liver mass. However, only 25% of the cells were transduced in our hands, so 0.25% of the liver mass was transduced cells.

**Fig. 3.** In vivo hAAT production in dogs A1 and A2 after transplantation of transduced hepatocytes. The serum concentrations of hAAT were determined before and after transplantation of 3.8 × 10^9 and 6.4 × 10^9 in animals A1 (solid circles) and A2 (open triangles), respectively. Each sample was analyzed in duplicate.

**Fig. 4.** PCR analysis of liver genomic DNA from dog A1. Genomic DNA isolated from various portions of the liver 1.5 and 4.5 months after transplantation was analyzed by 30 cycles of PCR amplification using Neo primers. (A) Ethidium bromide staining of the amplified PCR products. (B) Autoradiogram of the DNA blot from A hybridized with a radiolabeled Neo DNA probe. Lanes: a, DNA from a normal dog; b–e, DNA from hepatocytes transduced with LNCAAT, maintained in vitro, and diluted with normal dog DNA prior to amplification; f, cultured hepatocyte DNA from biopsy specimen 1.5 months after transplant; g, DNA as in lane f diluted 1:10 with normal DNA prior to amplification; h, cultured hepatocyte DNA from biopsy specimen 4.5 months after transplant; i, DNA as in lane h diluted 1:10 with normal DNA prior to amplification; j, liver DNA isolated from the same lobe as hepatocytes in lane k; k, DNA as in lane j diluted 1:10 prior to amplification; l and m, liver DNA isolated from different lobes 1.5 months after transplant. The arrow depicts the 320-base-pair Neo fragment.
This general estimate is in qualitative agreement with the observed relative number of transduced cells detected by the PCR assay.

**DISCUSSION**

Recently, the demonstration that freshly isolated hepatocytes containing transgenic markers can be transplanted into congenic mice such that the majority of transplanted cells reside in the liver and continue to function indefinitely has paved the way for hepatic gene therapy. The purpose of these experiments was to scale up hepatocyte harvest and infection protocols in a large animal model to study autologous hepatocyte transplantation after in vitro gene transduction with retroviral vectors. The methods of hepatocellular transplantation in dogs have been addressed in this study. We found that hepatocytes can be directly transplanted through the portal vasculature without serious sequelae or detectable evidence of histopathologic changes in the animal. However, there may be additional difficulties with transfusion of cultured hepatocytes vs. freshly isolated hepatocytes. A component in the serum used to culture the cells seems to be responsible for these problems. Although extensive washing of the cells dramatically reduced this problem, there was still some evidence of acute side effects with hepatocellular transplantation. By spacing the cell infusions, it was possible to transplant ~2% of the original liver mass after in vitro manipulation. By monitoring portal vein pressures during transplantation, we have effectively excluded primary portal vein thrombosis as the primary cause of the shock. Whether the episode of shock represents an immunologic, endotoxic, or physiological reaction or disseminated intravascular coagulation is not yet clear. The clinical effects of transplantation may be secondary and specific to this animal model since dogs appear to be extremely susceptible to changes or alterations in splanchic circulation (17). However, most importantly, these effects were transient and there is no evidence of long lasting sequelae.

Similar to hepatocytes from other species, canine hepatocytes can be maintained in culture and transduced with a retroviral vector, and the transduced hepatocytes express and secrete large quantities of hAAT in *vitro*. The CMV promoter has been shown to be extremely active in primary hepatocytes (18) and 5-fold more transcripts originate from the CMV promoter than from the viral long terminal repeat (5). The peak concentration of serum hAAT in A1 is similar to that obtained in transplanted transgenic hepatocytes in the mouse (6). Although there is a limited period of expression of the transgene in *vitro* in the transplanted transplanted hepatocytes, results of the PCR analysis show that a similar number of transduced cells are still present in the liver 1.5 and 4.5 months after transplantation when no hAAT was detected in the serum. If the transplanted hepatocytes had not survived *in vivo*, it is conceivable that some DNA fragments could be taken up by the reticuloendothelial cells. Because nonhepatocyte cells and dead hepatocytes are not represented in cultured cells (4), the similar PCR signal from cultured hepatocyte DNA and liver tissue DNA (Fig. 4, lanes f, g, j, and k) demonstrate that the proviral DNA is present in living hepatocytes.

The observations that serum hAAT concentrations eventually fell but transduced hepatocytes remained viable strongly suggest that the transcription of the transgene is shut-off *in vivo*. Schmidt et al. (19) have demonstrated that when marker genes are placed under transcriptional control of the CMV promoter in transgenic animals, there is no transcription detectable in the liver. Interestingly, Scharffmann et al. (20) have shown that the CMV promoter may be active in fibroblasts in culture but is inactivated once these culture cells are implanted into the animal. The CMV promoter could be reactivated by explanting and reculturing the transplanted tissue. We were unable to detect hAAT in the culture supernatants of hepatocytes taken from A1 after hAAT could not be detected in serum, suggesting that the promoter shut-off is not completely reversible in hepatocytes (data not shown). The issue of promoter shutoff in the liver can be resolved by using promoters derived from liver-specific cellular genes that are highly expressed under normal *in vivo* conditions. Some of these promoters such as albumin (21) and transthyretin (22) have been demonstrated to function in livers of transgenic mice. The maximal level of hAAT achieved in this study is two orders of magnitude less than what would be considered therapeutic and this approach will have to be improved upon for the treatment of hAAT deficiency in humans. However, with the development of technologies for large-scale hepatocyte isolation, retroviral transduction, and transplantation procedures in a large animal model, the strategy of hepatocyte transduction followed by autologous transplantation offers an approach to genetically correct many hepatic deficiencies in humans.

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