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Genetics

Adenovirus-mediated urokinase gene transfer induces liver regeneration and allows for efficient retrovirus transduction of hepatocytes *in vivo*

(gene therapy/α1 antitrypsin/retroviral and adenoviral vectors)

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ABSTRACT **Retrovirus-mediated gene transfer into hepatocytes *in vivo* results in long-term gene expression. Limitations include the need to remove two-thirds of the liver and the relatively low frequency of gene transfer. To increase gene transfer without surgical hepatectomy, mouse hepatocytes were transduced *in vivo* with a recombinant adenovirus that transiently expressed urokinase, resulting in high rates of asynchronous liver regeneration. During the regenerative phase, *in vivo* retroviral-mediated gene transfer in hepatocytes resulted in 5- to 10-fold greater transduction efficiencies than that obtained by conventional partial hepatectomy. In 3–4 weeks, the architecture and microscopic structure of the recipient livers were normal. The two-viral system of achieving permanent transgene expression from hepatocytes *in vivo* offers an alternative approach to current *ex vivo* and *in vivo* gene-transfer models.**

Permanent gene transfer and expression are of great importance when considering curative therapies for genetic disorders. Long-term gene expression has been accomplished by using retroviral gene transfer into hepatocytes with both *ex vivo* and *in vivo* methods (1–11). The transduction rate after retroviral gene transfer into hepatocytes *in vivo* is disappointingly low, and expression of therapeutic genes has not been sufficient for any cure of genetic disorders in animal models or humans (9–11). Efficient retrovirus integration into the host-cell genome requires the active proliferation of target cells with DNA replication and nuclear membrane breakdown during mitosis (12). Under normal physiological conditions at any given time, only 0.005% of hepatocytes divide (13). For retrovirus liver transduction, hepatocyte proliferation induced by surgical partial hepatectomy must occur on or about the time of retroviral delivery.

Due to the synchrony of hepatocyte division after partial hepatectomy, retroviral transduction can be performed only for a restricted period, and the amount of virus administered is limited by the volume of material that can be infused. The general approach has been to inject virus directly into the portal vasculature (4) or use an asanguineous perfusion technique (6). In mouse (4) and dog (10, 14) models, under the best circumstances ≈1% of hepatocytes can be transduced by *in vivo* delivery. The rat appears to be an anomalous organism in this regard because 5%–15% of hepatocytes can be transduced by these approaches (5, 7, 15).

In contrast to retrovirus, recombinant adenovirus can transfer a nonintegrated genome into nondividing hepatocytes with 100% efficiency but results in transient gene expression (16). In this study, the combined advantages of both vector delivery systems were used to increase permanent gene transfer in mouse hepatocytes *in vivo*. The mice were transduced with a recombinant adenoviral vector that expresses urokinase-type plasminogen activator (uPA) and creates a functional liver deficit subsequently resulting in a stimulus for liver growth. This strategy was based on a model of liver regeneration originally described in a transgenic mouse model producing hepatic urokinase (17). After adenovirus-mediated liver regeneration, it was possible to transduce hepatocytes *in vivo* with recombinant retroviral vectors at efficiencies higher than that achieved with partial hepatectomy.

MATERIALS AND METHODS

Recombinant Viruses. *Adenovirus.* The 1.326-kb *Hin*dIII/Asp-718 uPA cDNA fragment that only contains the protein-coding sequence was inserted into the *Hin*dIII/Asp-718 sites of pXCJL.1 (18) under transcriptional control of the Rous sarcoma virus (RSV) long terminal repeat promoter and upstream of the bovine growth hormone polyadenylylation signal (19). The virus was prepared after cotransfection with pJM17 (20) and the vector called Ad.RSV–uPA. Three days after infection of plaque-purified virus on 293 cells, the supernatant was tested for uPA antigen by ELISA and enzymatic activity by fibrin plaque assay (21). Construction of Ad.RSV–human α1 antitrypsin (hAAT) (22) and Ad.RSV–βGal (23) and their preparation, purification, and manipulation were as described (24).

Retrovirus. LNAlbhAAT and LBgeo retroviral vectors have been described (4). Recent characterization of the LNAlbhAAT vector showed that a portion of the albumin promoter had been deleted and that a 0.8-kb promoter-enhancer fragment remained in the vector (unpublished data). The retrovirus titers (208F cells) were 1×10^6 colony-forming units/ml for LBgeo vector (4) and 2×10^6 colony-forming units/ml for LNAlbhAAT vector. The titer of LNAlbhAAT vector was ≈10-fold less than the original clone used and reported (4). Maintenance of retroviral packaging cell lines and viral harvest were as described (4), except the final Polybrene concentration was 12 μg/ml.

Animals. All animal studies were done in accordance with the institutional guidelines of the University of Washington. C57BL/6 female mice aged 5–6 weeks were purchased from The Jackson Laboratory and housed in a specific pathogen-free environment. Blood samples were obtained by retroorbital bleeding. Mice were anesthetized by an i.p. administration of 0.5 ml of Avertin (20 mg/ml) (2,2,2-tribromoethanol). The full description of the portal vein cannulation will be described elsewhere. Briefly, a 3-cm silicone tube [0.02 in i.d., 0.037 in o.d. S/P medical grade, Baxter (Deerfield, IL) (1 in = 2.54 cm)] was inserted, and an adhesive (Histoacryl Blau; Braun, Melsungen, F.R.G.) was used to secure the tip in the

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Abbreviations: uPA, urokinase-type plasminogen activator; PT, prothrombin time; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; SGPT, serum glutamic pyruvic transaminase; RSV, Rous sarcoma virus; hAAT, human α1 antitrypsin; LTR, long terminal repeat.

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portal vein. The distal end of the cannula was placed in a subcutaneous pocket. In some mice the portal vein cannulation was done together with a two-thirds hepatectomy, as described (4). Adenovirus was diluted in 150 μl of Dulbecco's minimal essential medium (DMEM) (final volume) and injected over 5–10 min, and retrovirus was infused over 50 min via the cannula with a syringe pump.

Biochemical Measurements. The ELISA assay for uPA is based on two different monoclonal antibodies (H. Will, Max Delbrück Center, Berlin). One of the monoclonal antibodies was labeled with peroxidase according to the manufacturer's specifications (Pierce). The ELISA had a linear range from 1 ng/ml–50 ng/ml. hAAT concentrations in serum samples were determined by an ELISA assay as described (4). A Sigma diagnostic kit was used for colorimetric determination of the activity of serum glutamic pyruvic transaminase (SGPT) using 10 μl of serum (Sigma procedure no. 505). Prothrombin time (PT) was determined by a Sigma diagnostic kit (procedure no. T7280) with 100 μl of plasma from citrated blood in the presence or absence of aprotinin at 20 μg/ml.

Morphologic Assessments. For histological analysis, liver samples were fixed in 10% (vol/vol) neutral formalin, embedded in paraffin, and stained with hematoxylin/eosin. Autoradiography was done on 6-μm sections that were dip-coated with Kodak NTB-2 emulsion diluted 1:1 (vol/vol) with water and developed after a 2-week exposure. All slides were counterstained with hematoxylin/eosin, and at least 1000 cells from random fields were counted.

Thinly cut liver sections were analyzed for β-galactosidase, as described (25), or liver was fixed in OCT, frozen, and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (4). Hepatocyte isolation, culture, and X-Gal staining were done as described (26). The proportion of blue cells were determined from random fields; at least 600 cells were counted per plate.

RESULTS

Biochemical Effects of Ad.RSV–uPA Hepatic Gene Transfer. To induce liver regeneration, a recombinant adenoviral vector that expresses human urokinase from the RSV-long terminal repeat (LTR) promoter, Ad.RSV–uPA, was constructed. Human uPA can activate plasminogen across species (27). For comparison with Ad.RSV–uPA administration, control studies were done on mice that received an irrelevant control adenovirus or two-thirds partial hepatectomy. After infusion of 0.5×10^{10} plaque-forming units of adenovirus, enough to transduce 90% of hepatocytes (28), or partial hepatectomy, blood samples were analyzed at different times for serum urokinase concentrations, SGPT (an indicator of hepatocyte damage), and PT.

Infusion of Ad.RSV–uPA resulted in transient elevations of serum urokinase, reaching a peak value of 350 ng/ml (70–100 times greater than endogenous levels) 4 days after adenovirus administration, before falling to background concentrations by day 12 (Fig. 1). The rise in uPA was associated with an increase in serum SGPT concentrations, which reached 800 units/ml on day 6 (nl range 20–40 units/ml) before beginning to decline on day 9. Minimal elevation in serum SGPT (2- to 3-fold greater than normal) was found in Ad.RSV–hAAT-treated animals.

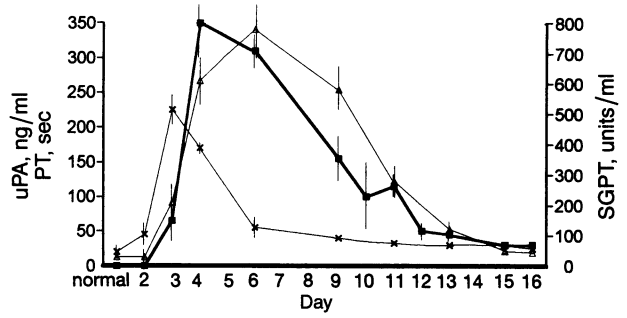

FIG. 1. Effects of Ad.RSV–uPA-mediated hepatic gene transfer. Serum concentrations of uPA (■), PT (x), and SGPT (▲) at different times after Ad.RSV–uPA administration (n = 5 per point). Vertical lines represent SD.

The PT measured as a means to estimate coagulation status in Ad.RSV–uPA recipient animals reached >200 sec on day 3 but fell to the normal range (25–35 sec) between days 11 and 13, whereas all control animals had normal PTs (Fig. 1). The observed disorders in clotting function in Ad.RSV–uPA-transduced mice resulted from the elevated plasma uPA activity and not from decreased synthesis of clotting factors by the liver because the PT done in the presence of aprotinin (a plasmin inhibitor) was normal (35 sec) (data not shown). Additionally, these animals had normal serum albumin and total protein concentrations. The presence of functional hepatic clotting factors (some of which have very short half-lives) and normal concentrations of serum proteins suggested that significant liver biosynthetic functions continued after urokinase-induced hepatocellular damage.

Histopathologic Liver Changes in Ad.RSV–uPA-Treated Mice. To begin to unravel the pathogenesis of Ad.RSV–uPA administration, histological evaluations were done on livers of experimental and control animals. The livers of animals that received Ad.RSV–uPA (but not control adenovirus) had a white appearance on days 3–7 after adenovirus administration, similar in gross morphology to that seen in the urokinase

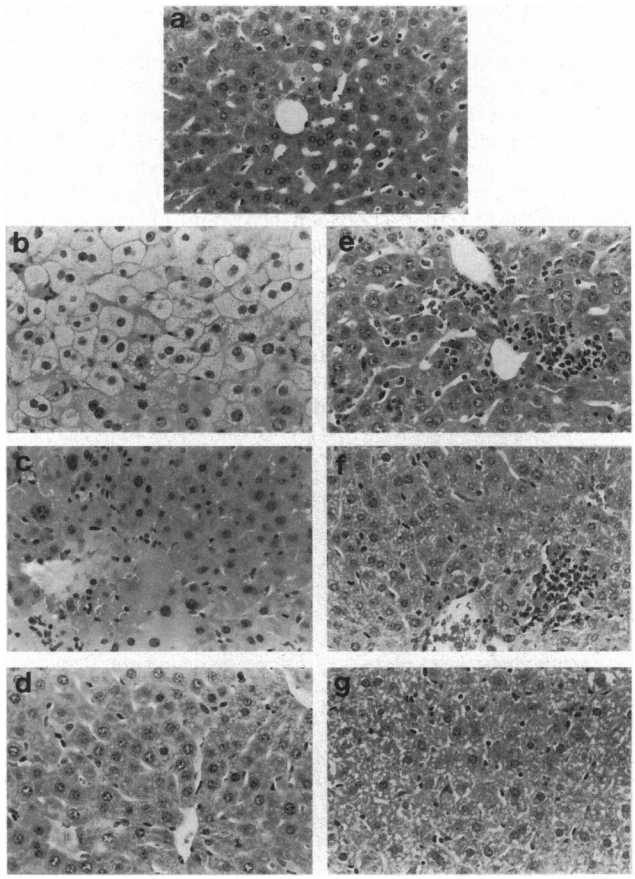

FIG. 2. Histology of livers from animals after adenovirus-mediated gene transfer. On day 0, mice were infused with 5×10^9 plaque-forming units of Ad.RSV–uPA or Ad.RSV–hAAT (control) adenovirus. At different periods, liver sections from recipient mice were stained with hematoxylin/eosin. (*a*) Normal, noninjected mouse liver. (*b–d*) Ad.RSV–uPA at days 4, 10, and 30, respectively. (*e–g*) Ad/RSV/hAAT on days 4, 11, and 30, respectively. Hepatocyte degeneration is visible in *b* and *c*; inflammatory infiltrates are seen in *e* and *f*. (×60.)

transgenic mouse model (17). Fourteen days after virus administration the livers appeared normal. Histological examination of other tissues—including spleen, kidney, stomach, intestines, lung, heart, and ovaries—were normal 3 to 4 days and 90 days after Ad.RSV–uPA transduction.

Liver histology from animals treated with recombinant adenovirus is presented in Fig. 2. Liver tissue from a normal mouse is shown in Fig. 2*a*. By days 3–4 after Ad.RSV–uPA administration, degeneration in ≈90% of the hepatocytes was observed (Fig. 2*b*). Eight to 10 days after Ad.RSV–uPA administration there was evidence of hepatic recovery, including the presence of multifocal regeneration (mostly in the periportal spaces) and heterogenous nuclei size (Fig. 2*c*). By 3–4 weeks, the liver appeared normal (Fig. 2*d*).

In contrast, animals receiving a control adenovirus had no evidence of generalized hepatic necrosis and hepatocyte degeneration. A pronounced inflammatory infiltrate mostly localized to the periportal spaces was observed on days 3–5 (Fig. 2*e*) and days 11–13 (Fig. 2*f*) but was resolved by 3 weeks (Fig. 2*g*). Inflammatory reactions have been seen with adenovirus administration that has been associated with an immunological response directed against adenoviral-transduced cells due to low-level production of adenovirus antigens in transduced cells (16).

Ad.RSV–uPA-Mediated Gene Transfer Leads to Hepatocyte Regeneration. At various times after adenovirus infusion, animals were infused with [^{3}H]thymidine, and autoradiography was done to establish that hepatocyte regeneration occurred after Ad.RSV–uPA administration. Representative examples are shown in Fig. 3, and the percentages of labeled parenchymal and inflammatory cell nuclei are summarized in Table 1. Thirty-four to 52% of the hepatocyte nuclei from Ad.RSV-uPA-treated mice were labeled over days 3–11 after transduction (Fig. 3*c*, Table 1). A sharp peak of hepatocyte regeneration (70%) at only 48 hr after partial hepatectomy (Fig. 3*b*) was seen, in agreement with previous studies (29). In contrast, the percentage of labeled hepatocytes was much lower in the control adenovirus-treated animals (Fig. 3*d*), ranging from 11% to 4% between days 3 and 14. This low level of hepatocyte labeling was similar to that found recently by Yang *et al.* (16). In total, these studies show that urokinase expression from hepatocytes induced significant liver parenchymal cell regeneration that lasted for 8 days.

Although the portion of inflammatory cells radiolabeled was similar in Ad.RSV–uPA and control adenovirus-treated animals, ≈10-fold more inflammatory cells appeared in the livers of control adenovirus-treated animals compared with Ad.RSV–uPA-treated animals (Fig. 2). Because [^{3}H]thymidine was infused over a 1-day period, the inflammatory cells may have been labeled before taking residence in the liver.

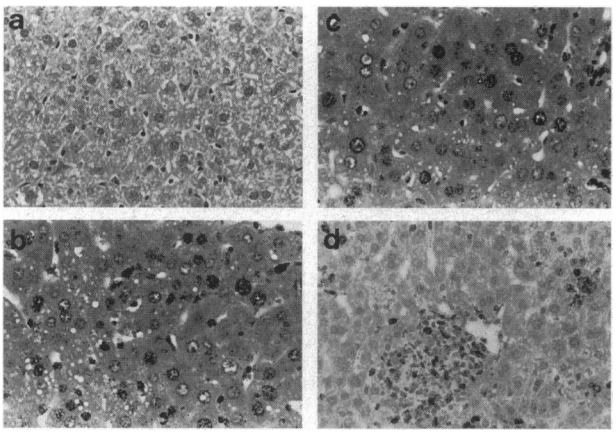

FIG. 3. Hepatocyte proliferation by autoradiographic analysis. Liver sections from mice described in Fig. 3 were autoradiographed and counterstained with hematoxylin/eosin. (*a*) Normal mouse liver. (*b*) Liver of partially hepatectomized (two-thirds) animal, 48 hr after the operation. (*c*) Ad.RSV–uPA adenovirus, day 4. (*d*) Ad/RSV–hAAT (control) adenovirus, day 4. *b* and *c* show most labeling in hepatocyte nuclei; *d* shows most labeling in inflammatory cells with rare hepatocyte labeling. (×60.)

Table 1. Hepatocyte regeneration in adenovirus-treated animals

Treatment	Labeled hepatocyte nuclei, %	Labeled inflammatory cell nuclei, %
Ad.RSV–uPA		
Day 3	49.3 (7.0)	64.8 (8.1)
Day 4	52.0 (5.4)	66.4 (8.7)
Day 5	48.6 (2.9)	38.3 (7.4)
Day 7	46.8 (5.3)	8.3 (3.6)
Day 9	34.3 (3.2)	18.1 (8.9)
Day 11	37.5 (5.0)	28.2 (7.5)
Day 14	12 (8.5, 15.5)	13.3 (15.2, 11.4)
Ad.RSV–hAAT (control)		
Day 3	11.2 (5.3)	57.8 (9.5)
Day 4	10.6 (2.7)	71.4 (11.9)
Day 5	6.4 (4.8)	49.0 (7.3)
Day 7	8.8 (0.9)	8.0 (4.9)
Day 9	9.4 (3.0)	19.7 (7.8)
Day 11	4.2 (1.3)	35.0 (9.5)
Day 14	3.3 (2.5, 4.1)	12.8 (8.4, 17.2)
Partial hepatectomy		
Day 2	69.8 (7.8)	2.8 (1.2)

One thousand hepatocyte and nonhepatic inflammatory cell nuclei were counted and scored for the presence or absence of silver grains. Mean values are listed as percentage of labeled cells (n = three or four animals per analysis, except at day 14, where n = 2 for both adenoviruses). The numbers in parentheses represent the SDs between animals, except on day 14, where the numbers represent the range of two experiments.

Ad.RSV–uPA Administration Allows for Permanent Retroviral-Mediated Gene Transfer *in Vivo*. The adenovirus-induced liver-regeneration model was used to introduce retroviruses into regenerating hepatocytes. One milliliter of recombinant retrovirus containing the hAAT cDNA (LNAlbhAAT) was infused into the portal vein of mice at different times after Ad.RSV–uPA, control adenovirus administration, or partial hepatectomy. Some mice received multiple infusions of retrovirus. The quantity of gene product and the relative amount of hepatic gene transfer were determined by periodic measurements of serum hAAT (Fig. 4).

Partial hepatectomized animals injected with LNAlbhAAT retrovirus had constitutive hAAT serum concentrations that varied between 20 and 100 ng/ml in five out of the first six recipients (Fig. 4*a*). One animal had an unusually high persistent serum hAAT concentration of ≈400 ng/ml after partial hepatectomy. To determine whether the high rate of gene transfer seen with partial hepatectomy in the one mouse could be reproduced, six additional mice were treated similarly, and all of these animals had serum hAAT concentrations in the 20–100 ng/ml range (data not shown). In general, the concentration of serum hAAT in partial-hepatectomized mice that received LNAlbhAAT vector was lower in this study than reported previously (4) and was most likely the result of using a packaging cell line with a 10-fold-lower titer. Retroviral-mediated gene transfer only occurred during a small window at ≈48 hr after partial hepatectomy in agreement with a previous study (4), and multiple infusions of retrovirus after partial hepatectomy did not increase gene transfer (data not shown).

The mice infused with a single dose of LNAlbhAAT retrovirus 3 days after Ad.RSVuPA administration had constitutive levels from 10 to 100 ng/ml in individual animals (Fig. 4*b*), whereas the mice that received three retrovirus (LNAlbhAAT) infusions on days 3, 5, and 7 after Ad.RSV–uPA had constitutive hAAT levels in the 100–400 ng/ml range (Fig. 4*c*). Because of the

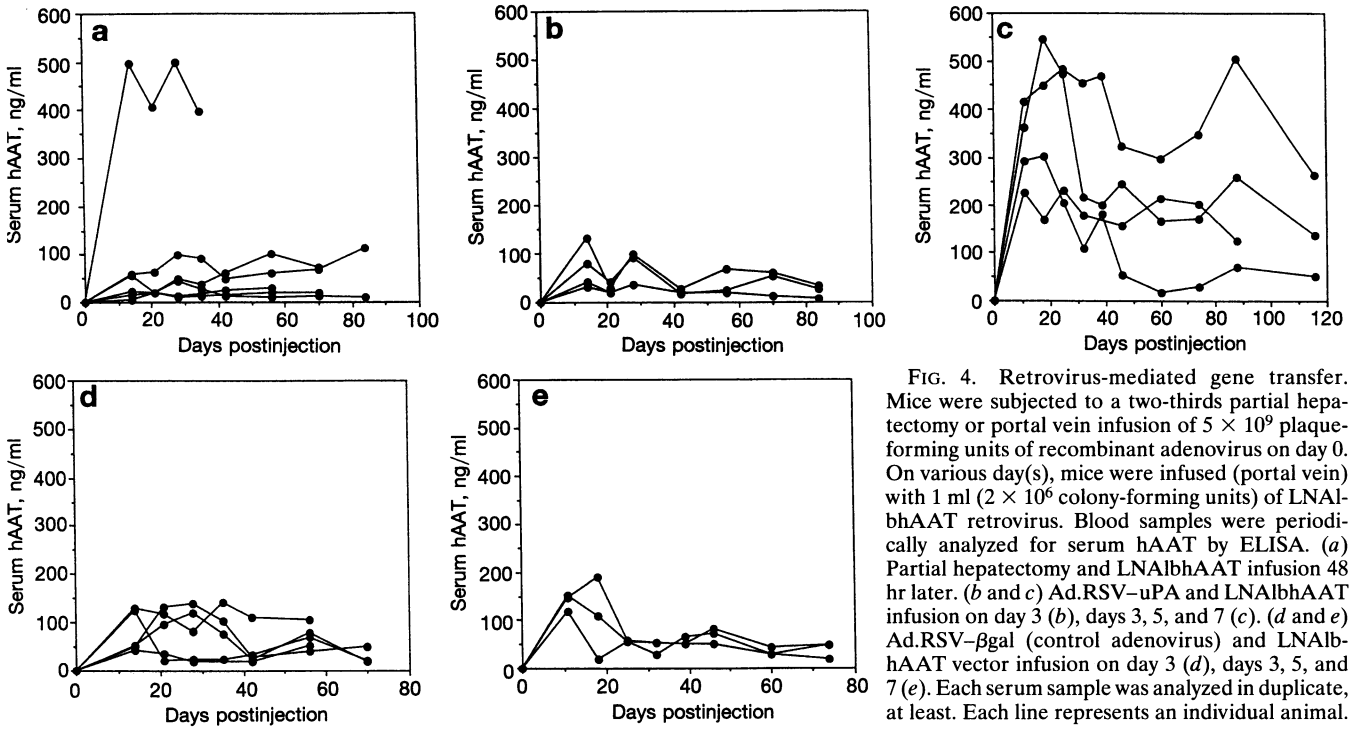

FIG. 4. Retrovirus-mediated gene transfer. Mice were subjected to a two-thirds partial hepatectomy or portal vein infusion of 5×10^9 plaque-forming units of recombinant adenovirus on day 0. On various day(s), mice were infused (portal vein) with 1 ml (2×10^6 colony-forming units) of LNAlbhAAT retrovirus. Blood samples were periodically analyzed for serum hAAT by ELISA. (*a*) Partial hepatectomy and LNAlbhAAT infusion 48 hr later. (*b* and *c*) Ad.RSV–uPA and LNAlbhAAT infusion on day 3 (*b*), days 3, 5, and 7 (*c*). (*d* and *e*) Ad.RSV–βgal (control adenovirus) and LNAlbhAAT vector infusion on day 3 (*d*), days 3, 5, and 7 (*e*). Each serum sample was analyzed in duplicate, at least. Each line represents an individual animal.

continued uPA production at the earlier time (e.g., 3 days), some hepatocytes transduced with retrovirus probably still contain adenovirus (that produced uPA) and were eventually destroyed. This hypothesis may explain why gene expression in recipient animals was >3-fold greater after three infusions compared with recipients that received a single infusion on day 3. In general, gene expression, as determined by the quantity of the hAAT serum marker, was >5-fold greater with the Ad.RSV–uPA-mediated regeneration than with that obtained with partial hepatectomy. Serum hAAT concentrations from mice infused with a control adenovirus before LNAlbhAAT infusion(s) were similar to mice that had been partially hepatectomized (Fig. 4 *a, d,* and *e*).

To prove definitively that gene transfer resulted in hepatocyte transduction, mice ($n = 4$ per group) underwent either partial hepatectomy or infusion of adenovirus and were then injected via the portal vein with the LBgeo retroviral vector, which expresses *Escherichia coli* β-galactosidase. Three weeks later, a small portion of the liver was removed for histochemical X-Gal staining (Fig. 5). From the remaining liver of two mice from each group, hepatocytes were isolated, cultured, and stained with X-Gal. The proportion of blue hepatocytes was determined from the population of cultured hepatocytes, and data were in excellent agreement with the proportion of β-galactosidase-positive hepatocytes detected on representative frozen sections (Fig. 5 *a–c*). In animals that received Ad.RSV–uPA and LBgeo vectors the proportion of β-galactosidase-positive hepatocytes in culture varied from 7 to 7.5%. In partial hepatectomized animals the proportion of β-galactosidase-positive cells was 0.8%, whereas liver sections from animals that received a control adenovirus before LBgeo vector infusion had <0.8% β-galactosidase-positive hepatocytes (Fig. 5*c*). Rare nonparenchymal β-galactosidase-positive cells were detected in these animals. In agreement with previous studies (4), no β-galactosidase-positive cells were found in the liver of normal mice that received LBgeo vector alone.

Interestingly, histochemical X-Gal staining of liver sections from Ad.RSV–uPA- and LBgeo-treated mice showed some single blue cells and clonal populations of blue cells of up to six blue cells per clone (Fig. 5*d*). This result suggests that at least two to three cell divisions occurred from the time of retrovirus transduction. The transduction efficiencies with the LBgeo vector agreed well with the relative quantities of serum hAAT using LNAlbhAAT vector and confirm that retroviral-mediated gene transfer was greater with the urokinase-induced hepatic regeneration than with partial hepatectomy.

DISCUSSION

The model system devised here takes advantage of the properties of two different viral vectors to achieve persistent gene transfer. The hypothesis of using a high-efficiency expression vector to transiently produce a gene product *in vivo* that will increase uptake of a second vector may have general applicability as additional gene transfer systems are developed. Recombinant adenovirus vectors infect 100% of hepatocytes, and Ad.RSV–uPA delivery induced asynchronous hepatocyte de-

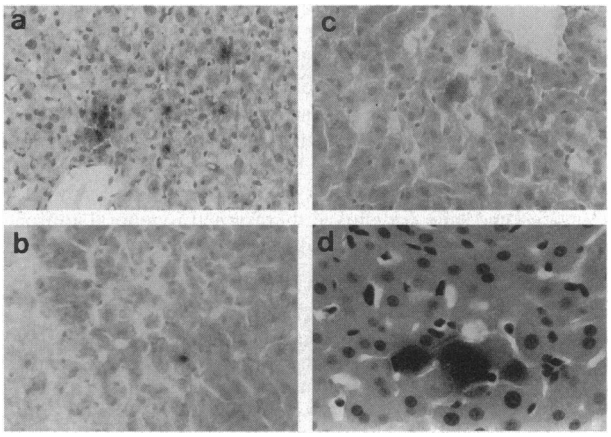

FIG. 5. Histochemical identification of retroviral-transduced hepatocytes. Mice were transduced with the Ad.RSV–uPA (*a* and *d*) or Ad/RSV–hAAT (*b*) control adenovirus and then with 1 ml of LBgeo vector on days 3, 5, and 7. For comparison, mice were infused with LBgeo vector 48 hr after partial hepatectomy (*c*). Three weeks later frozen (*a–c*) or fresh (*d*) liver sections were stained with X-Gal. A single clone of two β-galactosidase-positive hepatocytes is shown in *c*, whereas a single positive hepatocyte is present in *b*. Approximately 7.5%, <1%, and <1% of the hepatocytes stain blue in *a, b,* and *c*, respectively. [×60 (*a–c*); ×110 (*d*).]

generation in a majority of hepatocytes. The prolonged proliferation stimulus increased retroviral-mediated gene transfer by 5- to 10-fold over previous studies in mice using conventional methods (2, 4). It is important to note that this result was accomplished without a surgical partial hepatectomy. The transduction efficiencies obtained in this study are close to those needed to treat a number of hepatic enzyme deficiencies.

Urokinase is involved in diverse physiological and pathological processes including fibrinolysis, ovulation, and inflammation (30). The mechanism(s) of hepatocyte destruction and regeneration stimulation with ectopic expression of urokinase most likely involves the intracellular activation of hepatocyte-derived plasminogen. The plasmin protease activity is directed against a broad spectrum of proteins: matrix proteins (laminin, fibronectin), enzymes, clotting factors, or growth factors (30, 31), ultimately leading to hepatocellular death. Other explanations are possible. Secreted uPA may activate receptor-bound plasminogen (30). uPA and plasmin can change the hepatocyte microenvironment by altering the extracellular matrix and/or proteolytic activation of hormones and growth factors like hepatocyte growth factor (32, 33) that would directly stimulate hepatocyte proliferation. The mechanisms proposed are not mutually exclusive.

After intraportal infusion, adenovirus is taken up to a lesser extent by nonhepatic tissues. Because uPA expression from other tissues in transgenic mouse models is nontoxic (17), it will not limit the application described here. The major obstacle to this approach was bleeding that resulted from transient elevation in plasma uPA concentrations after Ad.RSV-uPA administration (17, 34). We have recently modified the urokinase cDNA such that a recombinant adenovirus produced a protein that, when expressed in hepatocytes, stimulated liver regeneration but was not secreted into the bloodstream (37).

Although the gene-transfer strategy developed in this study caused transient hepatocellular injury, full recovery occurred within a few weeks. Furthermore, there was no evidence of hepato-biosynthetic deficiencies after Ad.RSV-uPA administration. The unaffected liver status after Ad.RSV-uPA gene transfer is a main advantage of our model in relation to the uPA transgenic mouse regeneration model, in which the mice develop fatal liver insufficiency.

The ability to induce asynchronous liver regeneration with a recombinant adenovirus that subsequently allows for retrovirus transduction may have value in larger animal models where, unlike the mouse model, size will not limit the ability to perform multiple infusions of retrovirus over several days for efficient hepatocyte transduction. The ability to concentrate (35) retroviral vectors to higher titers and determine optimal promoter sequences (8, 36) *in vivo* may also prove important in regard to the model of hepatic gene transfer described here. Clearly, this study serves only as a model and will need refinement before it can be considered for clinical use. As new-generation adenoviral vectors become safer for clinical therapies, this general approach may prove beneficial for genetic disorders where permanent gene transfer is necessary for a cure.

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