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ARTICLE

Liver Cirrhosis Is Reverted by Urokinase-Type Plasminogen Activator Gene Therapy

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Liver cirrhosis represents a worldwide health problem and is a major cause of mortality. Cirrhosis is the result of extensive hepatocyte death and fibrosis induced by chronic alcohol abuse and hepatitis B and C viruses. Successful gene therapy approaches to this disease may require both reversal of fibrosis and stimulation of hepatocyte growth. Urokinase-type plasminogen activator (uPA) may serve this function, as it is an initiator of the matrix proteolysis cascade and induces hepatocyte growth factor expression. In a rat cirrhosis model, a single iv administration of a replication-deficient adenoviral vector encoding a nonsecreted form of human uPA resulted in high production of functional uPA protein in the liver. This led to induction of collagenase expression and reversal of fibrosis with concomitant hepatocyte and improved liver function. Thus, uPA gene therapy may be an effective strategy for treating cirrhosis in humans.

Key Words: **experimental cirrhosis; hepatic regeneration; adenovirus; vector; gene therapy; metalloproteinases.**

INTRODUCTION

Liver cirrhosis represents a worldwide health problem and is a major cause of mortality. Cirrhosis is the common end for chronic alcohol abuse and hepatitis C and B virus infections (1) and no definitive cure is available so far. Alcoholism alone affects around 14 million people in the United States (2). The potential application of gene therapy protocols to human hepatic cirrhosis will depend on the successful and tissue-specific delivery of therapeutic genes to livers affected with extensive fibrosis. We reason that gene therapy applications to reverse the exacerbated fibrosis, typical major hallmark of cirrhotic livers, might be developed. Likewise, targeted delivery of genes promoting liver cell proliferation could favor the rapid reestablishment of functional hepatic mass. An adenoviral vector bearing a modified cDNA coding for a nonsecreted form of human urokinase plasminogen activator (Ad-ΔhuPA) was used to deliver the gene *in vivo* (kindly provided by Dr. Mark Kay) (3). The nonsecreted uPA was chosen to diminish the risk of bleeding, which would be particularly problematic in cirrhotic animals that may have preexisting coagulopathy. A huPA cDNA was used because it is known as a potent activator of latent hepatic collagenases (4) which in turn would promote the degradation of extracellular matrix deposited in the Disse's space (perisinusoidal space). Excess collagenous proteins hamper the flow exchange of nutrients between circulating blood and hepatocytes (4–6) and provoke liver dysfunction. Furthermore, nonsecreted uPA leads to rates of brisk hepatic liver cell regeneration in normal noncirrhotic animals similar to the unmodified or wild-type uPA, without the side effects related to bleeding tendency (3). The adenovector approach was chosen to quickly analyze the effects of potentially therapeutic genes, as adenovirus has been shown to be an efficient vector in cirrhotic liver models (7).

In this paper we report that one single injection of the adenoviral Ad-ΔhuPA through the iliac vein of severely cirrhotic rats can target liver tissue in a specific way and

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induce profound beneficial changes. We have been able to induce a significant reversion in CCl_4-induced hepatic fibrosis and at the same time stimulate liver cells to regenerate. Furthermore, an improvement in functional hepatic tests was evident.

MATERIAL AND METHODS

Animals. The experimental model consisted of Wistar rats undergoing chronic administration of CCl_4 (8), an animal model that closely resembles human hepatic cirrhosis induced by alcohol abuse or chronic infection with hepatitis C virus. Briefly, animals weighing 80 g received three doses a week via ip of a mixture 1:6 of CCl_4–mineral oil for the first week, a ratio of 1:5 the second week, 1:4 the third week, and 1:3 the fourth through eighth week. Control rats were pair-fed and injected with vehicle only. All animal studies were performed on male Wistar rats in accordance with the University of Guadalajara's animal guidelines. All experimental methods have been described previously (7). Adenovirus infusions were performed through the iliac vein.

Adenovirus vectors. Adenoviral plasmid pAd.PGK-ΔNΔC-huPA (pAd-ΔhuPA) cloning has been previously described (3) and is essentially a first-generation adenoviral vector bearing a modified cDNA coding for nonsecreted human urokinase plasminogen activator (Ad-ΔuPA). The preparations of this Ad vector were monitored for endotoxin and mycoplasm contaminants and were titered as previously described (9). The rationale for using this vector resided in the advantage of the nonsecreted uPA that does not cause hypocoagulation and spontaneous bleeding, which represents a major drawback in cirrhotic animals.

The Ad-GFP vector used here (irrelevant adenovirus) is an E1- and E3-deleted replication-defective adenovirus vector previously described (9). The vectors were produced at the Baylor College of Medicine Gene Vector Laboratory under Good Laboratory Practice conditions. The vectors were titered and characterized as described (9) and had a vector particles (vp) to infection units (IU) ratio of ≤30.

Preparation of liver homogenates. Rats were sacrificed at indicated times in Fig. 1a and liver homogenates were prepared from 150 mg of tissue as described (10) and kept at –70°C. Briefly, for uPA, in the presence of protease inhibitors, 150 mg of liver was homogenized in 400 μl of homogenization buffer (0.05 M Tris–HCl, 0.15 M NaCl, 0.01 M Hepes, 2 mM $CaCl_2$, 0.01% Tween 80, 1 mM phenylmethylsulfonyl fluoride, pH 8.5). One hundred microliters of such homogenates was used for ELISA. At the same time, serum samples (100 μl) were obtained and kept at –20°C until used for ELISA. Total protein levels in serum were determined using Bradford assay of protein quantification (11). For metalloproteinase-2 (MMP-2) assay, samples were homogenized using a high-speed mixer homogenizer (Politron PT 3000, Kinematica AG, Brinkmann, Switzerland) for 5 min at 8000*g* in 4 ml of 0.15 M NaCl at 4°C. After three freeze–thaw cycles, the homogenate of each sample was sonicated twice at 21 kilocyles per second for 1 min at 4°C and centrifuged at 8000*g* for 10 min at 4°C, aliquoted, and kept at –70°C until further use.

uPA and MMP-2 ELISA determinations. ELISAs were performed using a commercially available kit (Biopool TintElize uPA, Sweden) which is an enzyme immunoassay specific for quantitative determination of human uPA, with a detection limit of 0–4 ng/ml.

The Biotrak MMP-2 activity assay system from Amersham Pharmacia Biotech provides quantitative determination of MMP-2 in tissue homogenates and others (detection limit 0.75–12 ng/ml).

Biochemical assays. Blood was drawn from animals at specified times, and serum transaminases ALT, AST, alkaline phosphatase, and bilirubins were determined in an automated Sincron-7 analyzer at Hospital Civil de Guadalajara.

Histological examination and immunohistochemistry of liver sections. Rats were sacrificed at 2, 4, 6, 8, and 10 days after Ad-ΔhuPA administration (Fig. 1a). A group of cirrhotic animals injected with Ad-GFP (irrelevant adenovirus) and vehicle only as well as a group of normal animals were used as controls. Five rats were included for each group. For the histologic study, the liver was immediately removed and fixed by immersion in 10% paraformaldehyde diluted in phosphate–saline buffer (PBS), dehydrated in graded ethylic alcohol, and embedded in paraffin. Sections 5 μm thick were stained with hematoxylin/eosin and Masson trichrome. In these latter slides the percentage of liver tissue affected by fibrosis was determined using a computer-assisted automated image analyzer (Qwin Leica) by analyzing 10 random fields per slide and calculating the ratio of connective tissue to the whole area of the liver. For immunohistochemistry, liver sections were mounted in silane-covered slides and deparaffinized, and the endogenous activity of peroxidase was quenched with 0.03% H_2O_2 in absolute methanol. Liver sections were incubated overnight at room temperature with mouse monoclonal antibodies against PCNA and α-smooth muscle actin (Boehringer Mannheim, Germany) diluted 1/20 and 1/50, respectively, in PBS and with goat polyclonal antibodies against human uPA (Chemicon International, U.S.A.) diluted 1/400 in PBS. Bound antibodies were detected with peroxidase-labeled rabbit polyclonal antibodies against mouse or goat immunoglobulins and diaminobenzidine and counterstained with hematoxylin. For quantification, 10 random fields of intralobular and periportal areas were evaluated at ×400 magnification. Immunohistochemical positive and negative cells were counted by an automated image analyzer (Qwin, Leica) and the data expressed as percentages of positive cells. Histopathology was interpreted by two independent board-certified pathologists who were blinded to the study. There was a 5% margin of difference in their analyses.

Semiquantitative RT-PCR of cDNA. Total RNA was prepared immediately after liver was extracted at the time of sacrifice and RT-PCR was carried out essentially as described (12) with some modifications by Delgado-Rizo *et al*. (13). The following specific primers were used: 5′-ATGCTCATGGACCCTGGT-3′ (HGF sense primer), 5′GCCTG-GCAGCTTCATTA-3′ (HGF antisense primer), 5′-CAGTGATGATCT-CAATGGGCAAT-3′ (c-met sense primer), and 5′AATGCCCTCTTCCTAT-GACTTC-3′ (c-met antisense primer). Thirty cycles of PCR were performed, with the following conditions: denaturation for 5 min at 94°C, annealing for 1 min at 60°C, and elongation for 1.5 min at 72°C. Standardization of constitutive gene expression was accomplished with the HPRT gene.

RESULTS AND DISCUSSION

Cirrhosis was chemically induced as previously described (8). Mature rats (100–150 g) were injected ip with carbon tetrachloride (CCl_4) three times a week for 6 to 8 weeks. This effectively induced cirrhosis by 6 weeks (Fig. 1a). At the end of the sixth week, a single dose of 6×10^{11} vector particles/kg of Ad-ΔhuPA or Ad-GFP were injected into the iliac vein. The animals were sacrificed for analysis starting 2 days after injection. CCl_4 administration was continued such that rats sacrificed at days 8 and 10 received three additional injections of CCl_4.

Modified huPA Is Localized Mainly Intracellularly

Human uPA expression was detected by ELISA in treated animals. The level of expression averaged 4 ng/mg in liver homogenates (100 μl/liver) and 1 ng/ml in serum (100 μl) (Fig. 1b). Human uPA levels decreased after day 2 but showed clearly detectable levels throughout the study above the control animals injected with Ad-GFP. The human uPA protein detected in serum may be due to leakage from damaged hepatocytes or reflect a small amount of released protein after a burst of cellular expression. It is possible that this leaked uPA might be mediating activation of circulating plasminogen and/or that embedded in liver extracellular matrix, converting it

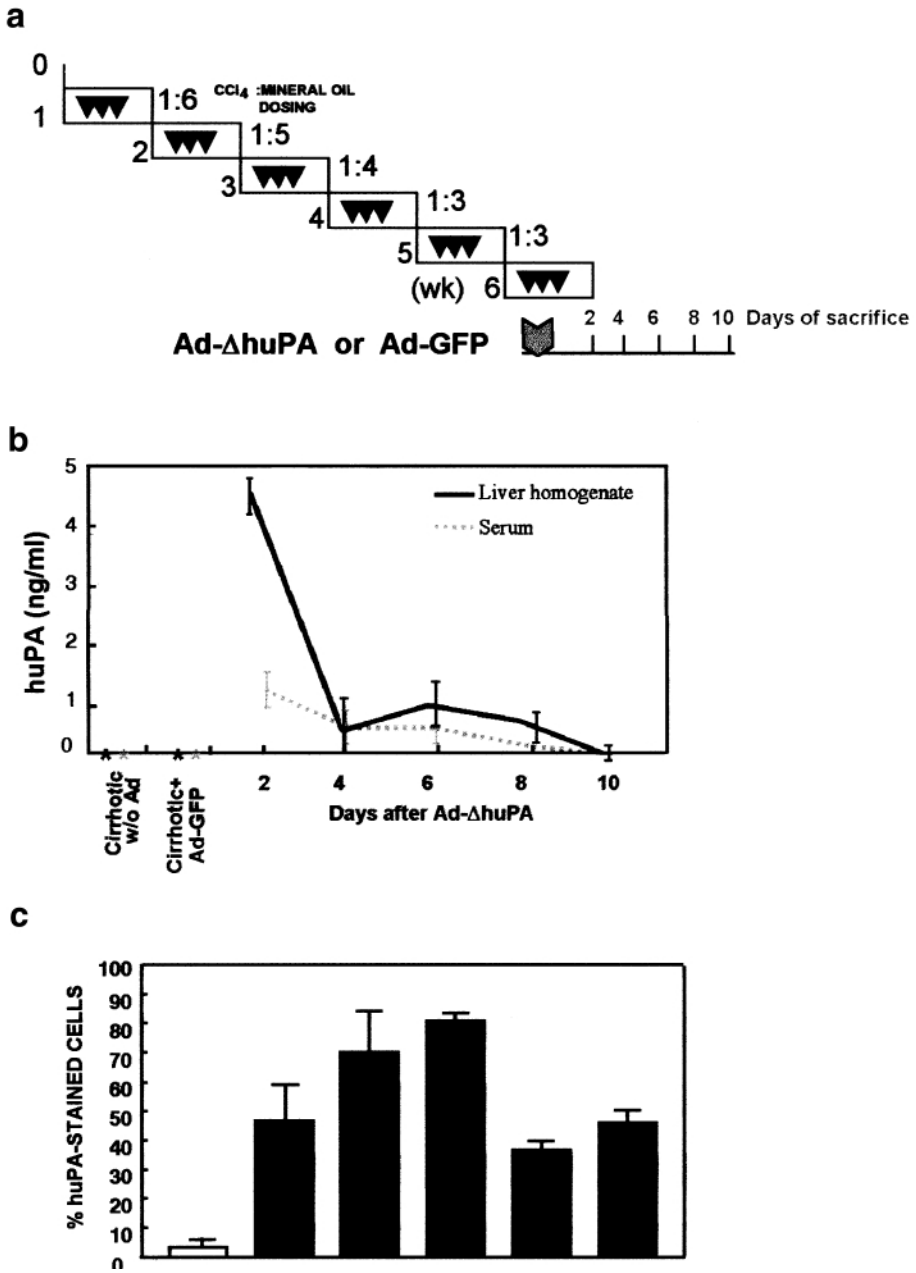

FIG. 1. (a) Schedule of CCl_4–mineral oil dosing, adenoviral vectors (Ad-GFP or Ad-ΔhuPA) administration (⇓), and days of sacrifice. (b) Liver homogenate and serum levels of modified human uPA induced by Ad-GFP or Ad-ΔhuPA adenoviral vector injection in CCl_4-treated rats. (—) huPA liver homogenate levels of rats that received a single gene injection ($n = 5$); (- - -) huPA serum levels of rats that received a single gene injection ($n = 5$). All values are presented as means ± standard deviation. (c) Percentages of huPA immunostained cells after huPA adenovirus vector administration. Quantification of positive and negative cells was done in four random fields by an automated image analyzer. Open bar, liver sections from cirrhotic untreated animals were used as controls.

to plasmin, and thus activating latent MMPs (14, 15), which in turn digest collagenous matrix. Human uPA expression was detected in 46–80% of hepatocytes in immunostained liver sections from Ad-ΔhuPA-injected rats starting on day 2 and peaking on day 6 (Fig. 1c). Kupffer cells and biliary epithelium also showed immunoreactivity.

Overexpression of Hepatic huPA Correlates with Induction of Metalloproteinases and Reversal of Fibrosis

Ad-ΔhuPA administration led to almost complete resolution of periportal and centrilobular fibrosis compared to a progressive fibrosis in controls (Figs. 2a and 2c).

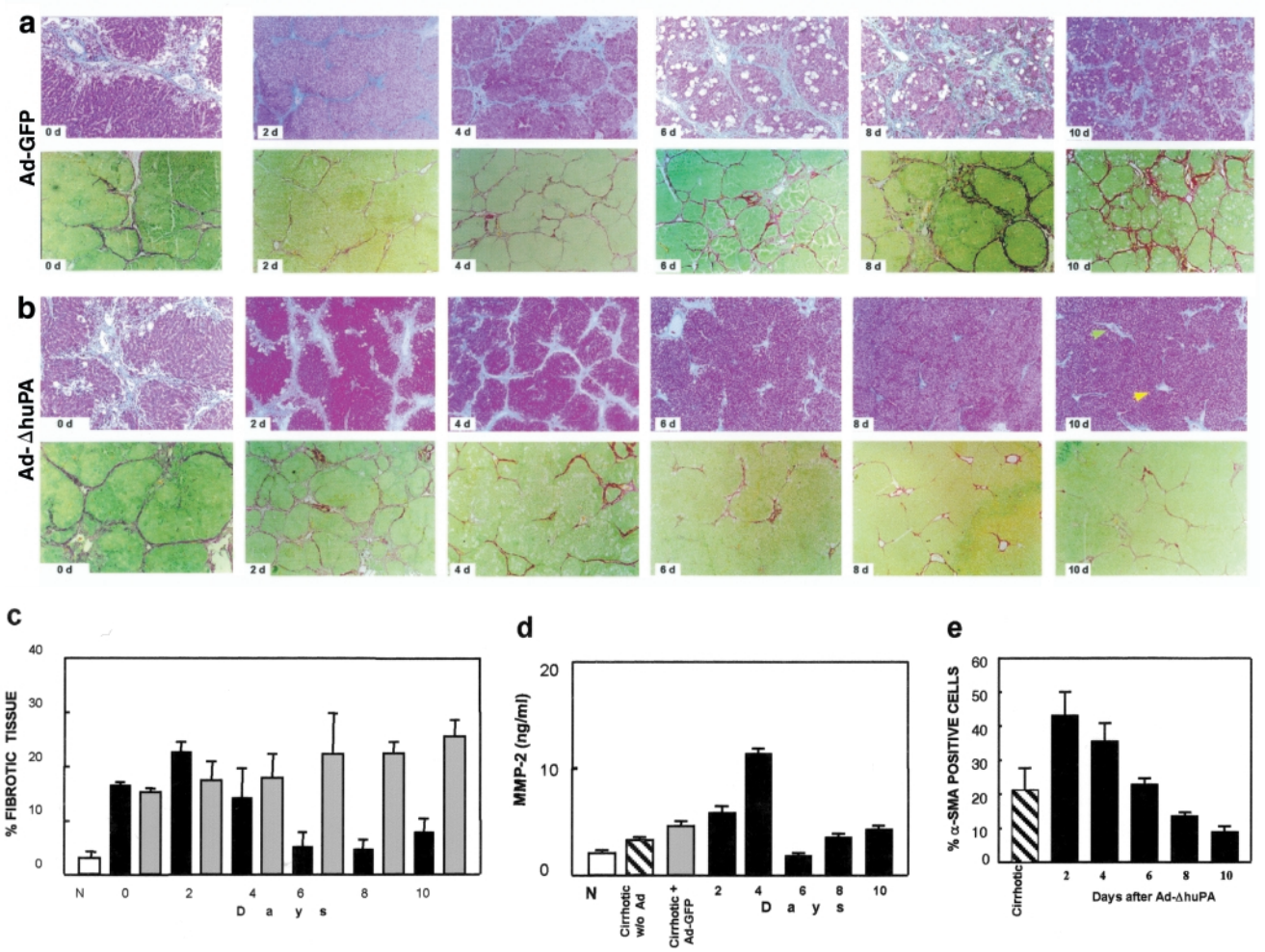

FIG. 2. (a and b) Liver sections of cirrhotic rats after CCl_4 administration and Ad-ΔhuPA or Ad-GFP adenoviral vector injection. (Upper panels) Masson trichromic staining (×50), (lower panels) Sirius red staining (×50). (a) Cirrhotic nodules are surrounded by thick fibrotic bands after 2, 4, 6, 8, and 10 days of Ad-GFP adenoviral vector injection showing progressive fibrosis. (b) In contrast, after 10 days of huPA adenoviral vector injection there are only thin fibrous bands extending from portal areas (green arrow) and mild central vein sclerosis (yellow arrow). (c) Percentages of fibrous tissue deposition after Ad-GFP or Ad-ΔhuPA adenoviral vector infusion and normal rats. Determinations of fibrous tissue were done in 10 random fields by an automated image analyzer. Open bar, normal rats; gray bars, rats treated with CCl_4 for 6 weeks with Ad-GFP adenoviral vector injection; black bars, rats treated with CCl_4 and injected with huPA adenoviral vector. N represents normal noncirrhotic rats. Values are presented as means ± standard deviation. (d) Determination of matrix metalloproteinase-2 (MMP-2) activity in liver homogenates by a specific ELISA of normal and cirrhotic rats treated with adenoviral vectors (Ad-GFP or Ad-ΔhuPA). Open bar, normal rats; crossed bar, liver sections from cirrhotic untreated animals; gray bar, cirrhotic animals treated with Ad-GFP adenoviral vector (after 4 days); black bars, cirrhotic animals treated with Ad-ΔhuPA adenoviral vector. N represents normal noncirrhotic rats. (e) Percentages of αSMA-immunostained cells after Ad-GFP or Ad-ΔhuPA adenoviral vector infusion. Quantification of positive and negative cells was done in four random fields by an automated image analyzer. Crossed bar, cirrhotic untreated rats; black bars, rats treated with CCl_4 and injected with huPA adenoviral vector. Values are presented as means ± standard deviation.

FIG. 3. (a) Hepatocyte regeneration as measured by PCNA staining. Mitosis in hepatocytes was assessed by immunohistochemistry using anti-PCNA antibody after Ad-GFP or Ad-ΔhuPA adenovirus vector injection. (Left panel) Hepatocytes with nuclear PCNA immunostaining after huPA adenoviral vector injection. Cirrhotic rats after 2 days have abundant PCNA-positive hepatocytes (red arrow) in coexistence with frequent mitotic figures (black arrows) and binucleated cells (blue arrows) (×320). Moreover, positive cells were detected at 4, 6, 8, and 10 days (×200). In comparison (right panel), few hepatocytes with PCNA immunostaining (×200) at 2, 4, 6, 8, and 10 days after Ad-GFP adenovirus vector injection are shown. (b and c) Percentages of PCNA-immunostained cells in periportal and lobular areas, respectively, after Ad-ΔhuPA- or Ad-GFP adenovirus vector injection. Quantification of positive and negative cells was done random field by an automated image analyzer. Liver sections from cirrhotic untreated animals were used as controls. Open bars, normal rats; gray bars, rats treated with CCl_4 for 6 weeks with Ad-GFP adenoviral vector injection; black bars, rats treated with CCl_4 and injected with huPA adenoviral vector. Values are presented as means ± standard deviation. (d) Determination of HGF and c-met gene expression by semiquantitative RT-PCR. Total RNA was extracted from livers at indicated times and cDNA was obtained. Standardization of constitutive gene expression was accomplished with the HPRT gene. (e) Prothrombin time of cirrhotic rats sacrificed at different days after Ad-ΔhuPA- or Ad-GFP adenoviral vector injection. Open bar, normal rats; gray bars, rats treated with CCl_4 for 6 weeks with Ad-GFP adenoviral vector injection; black bars, rats treated with CCl_4 for 6 weeks with Ad-ΔhuPA adenoviral vector injection. Dotted line represents normal prothrombin time. Values are presented as means ± standard deviation.

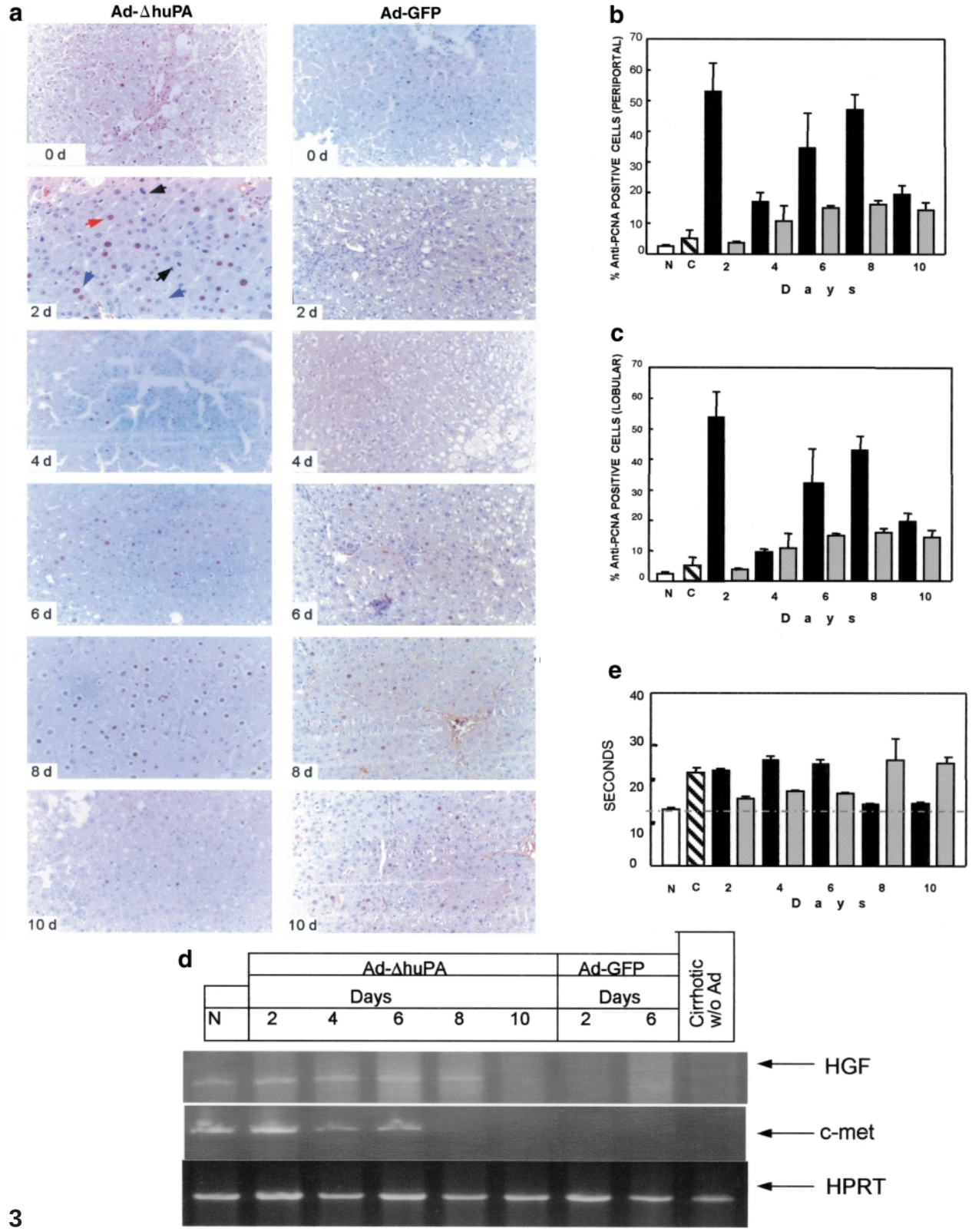

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TABLE 1
Liver Enzymes[a]

	Normal	Ad-ΔhuPA day2	Ad-GFP day2	Ad-ΔhuPA day8	Ad-GFP day8
ALT (IU.L)	73.6 ± 10.9	1563 ± 948.8	890 ± 260.2	88.5 ± 24.7	410 ± 45
AST (IU/L)	101.3 ± 30.9	1590 ± 957.09	737 ± 156.98	137 ± 29.7	2250 ± 55.6
Alkaline phosphatase (IU/L)	159.7 ± 34.7	525.7 ± 11.3	382.5 ± 40.3	205 ± 54.5	454 ± 46.5

[a] Measurements were performed at stipulated times in serum of cirrhotic rats after vector infusion.

Computer-assisted analyses showed that Ad-ΔhuPA-treated rats had an 85% reduction in hepatic fibrosis at day 10 (Figs. 2b and 2c). In addition, in cirrhotic livers with severe fibrosis, hepatic stellate cells (HSC) are increased in fibrotic areas, many of which have an activated phenotype identified by expression of α-smooth muscle actin (SMA) (1). Cirrhotic livers showed numerous α-SMA-positive cells embedded in the perinodular fibrous tissue which increased 2 days after Ad-ΔhuPA administration but decreased to 50% of the control cirrhotic livers by day 10 (Fig. 2e).

A potential mechanism for the degradation of fibrotic tissue observed with Ad-ΔhuPA is through the activation of latent metalloproteinases complexed with TIMPs. These MMPs are effector proteins downstream of uPA in the matrix proteolysis cascade. We found an increased expression of MMP-2 in liver homogenates of Ad-ΔhuPA-treated animals (Fig. 2d). This assay is a very sensitive ELISA, which detects minimal changes in levels of total MMP-2 (active and pro-MMP-2) (16), which specifically degrades collagen type IV and other collagens to a lesser degree (17, 18). We believe that most MMP-2 in Ad-ΔhuPA animals is in the form of active MMP-2. However, we need to further elucidate quantitative amounts of active MMP-2 and MMP-2 species complexed with its specific inhibitor, TIMP-1.

HuPA Induces a Brisk Liver Cell Regeneration

In addition to resolution of fibrosis, liver regeneration was observed in cirrhotic rats treated with Ad-ΔhuPA. Two days after Ad-ΔhuPA administration, liver sections contained a substantial increase in the number of mitotic figures, binucleated hepatocytes, and cells expressing proliferative cell nuclear antigen (PCNA) (Figs. 3a–3c). The percentage of PCNA-positive cells was still as high as 40% on day 8 and included periportal and lobular areas (Figs. 3b and 3c). The hepatocyte growth observed correlated with increased expression of hepatocyte growth factor (HGF) and its cognate receptor c-met measured by semiquantitative RT-PCR (Fig. 3d). It has been reported that HGF is produced by hepatic stellate cells. However, there is also evidence that Kupffer cells, biliary epithelial cells, and hepatocytes produce HGF (19, 20). We believe that this HGF mRNA upregulation is accounted for by these latter cells. HGF and c-met RNA were not detectable in control Ad-GFP-treated or untreated cirrhotic animals.

Ad-ΔhuPA gene therapy also led to improvement in hepatic function in cirrhotic animals (Fig. 3e). Although transduction with adenovirus led to an increase in transaminases at day 2 (Table 1), Ad-ΔhuPA-treated cirrhotic animals had a subsequent decline in liver enzymes reaching normal values by day 8. These important changes were accompanied by normalization of prothrombin time which was not observed in animals treated with Ad-GFP (Fig. 3e).

Thus, expression of uPA in cirrhotic livers led to resolution of fibrosis and regeneration of functional hepatocytes. Multiple mechanisms may account for the induction of hepatocyte proliferation in this system. Matrix degradation by activation of the metalloproteinases cascade may lead to remodeling of the distorted architecture and angiogenesis and may free up space for hepatocyte expansion. In addition, metalloproteinases may prime hepatocytes to be more responsive to growth factors (4). Increased HGF mRNA expression was observed, and uPA has been reported to activate single-chain HGF (scHGF, inactive form) to two-chain HGF (tc HGF, active form) (21–23). We believe that, in these experiments, HGF bound to matrix is released and then activated by uPA because of its structural similarity with plasminogen and related serine proteases as has been previously reported (24). In addition, expression of the c-met receptor for HGF was increased. Thus, degradation of fibrosis together with upregulation of c-met and activation of HGF may be the trigger for liver function recuperation in this model of cirrhosis.

Ueki and colleagues recently reported the use of an HGF-based gene therapy approach for liver cirrhosis (25). However, uPA gene therapy has several advantages over their approach. HGF infusion into normal rat liver has been reported to stimulate hepatocyte proliferation only in the periportal areas (26, 27). In contrast, pretreatment with collagenase led to increased hepatocyte–DNA synthesis in all lobular areas (27). In uPA-treated animals

reported here, liver cell proliferation was induced throughout the entire parenchyma as well as in periportal areas (Fig. 3). Induction of hepatocyte growth without establishment of normal architecture is not likely to restore liver function. Thus, we also observed a rearrangement of the hepatic architecture in the livers of Ad-ΔhuPA-treated cirrhotic rats. Then, the results presented here provide a potential new therapeutic approach for liver cirrhosis.

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