

# Hepatocyte Growth Factor Induces Hepatocyte Proliferation *In Vivo* and Allows for Efficient Retroviral-Mediated Gene Transfer in Mice

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Recombinant retroviral vectors are an attractive means of transferring genes into the liver because they integrate into the host cell genome and result in permanent gene expression. However, efficient *in vivo* gene transfer is limited by the requirement of active cell division for integration. Traditional approaches to induce liver proliferation have the disadvantage of inducing hepatocellular injury by delivery of toxins or by surgical partial hepatectomy. As a nontraumatic alternative, we show that exogenous hepatocyte growth factor (HGF) is a powerful and safe mitogen for the mature intact murine liver when delivered continuously into the portal vein. A 5-day infusion of human HGF (5 mg/kg/d) resulted in >140% increase in relative liver mass, which returned to normal in 4 to 5 weeks. This clearly shows that an exogenous growth factor can induce robust liver proliferation *in vivo*. In addition, we show that the HGF-induced proliferation was independent of interleukin-6, an essential cytokine involved in liver regeneration after partial hepatectomy. When recombinant retroviral vectors were infused in combination with HGF, 30% of hepatocytes were stably transduced with no indication of hepatic injury or histopathology. These results show the ability to obtain a clinically relevant transduction efficiency with retroviral vectors *in vivo* without the prior induction of liver injury. The level of hepatic gene transfer achieved has the potential to be curative for a large number of genetic liver diseases. (HEPATOLOGY 1998;28:707-716.)

The liver is an important target organ for gene therapy because a number of genetic diseases result from the absence or deficiency of a hepatocyte specific gene product. Gene

therapy to treat hereditary liver diseases requires efficient and permanent hepatic gene transfer. Retroviral vectors are especially attractive for this purpose because of their ability to integrate into the host cell genome, resulting in sustained gene expression.<sup>1,2</sup> In addition, they do not elicit a cellular immune response, because no viral genes are contained in the vector. An important limitation to retroviral-mediated gene transfer has been the requirement of active cell division.<sup>3</sup> Under physiological conditions most mature hepatocytes are quiescent<sup>4</sup>; therefore, several strategies have been employed to induce hepatocyte regeneration including surgical partial hepatectomy,<sup>5</sup> expression of toxic gene products with use of two viral vectors,<sup>6,7</sup> and portal branch ligation.<sup>8,9</sup> However, these approaches have the disadvantage of inducing hepatocellular injury, which would be difficult to justify in patients with nonlethal genetic diseases. A more acceptable method would involve the stimulation of hepatocyte proliferation by an exogenous mitogen without prior cell loss. The optimal mitogen should not only be a powerful stimulant of DNA synthesis in normal mature hepatocytes, but should also be safe, nonimmunogenic, and act in a dose-dependent manner.

The *in vivo* retroviral-mediated liver transduction efficiencies originally reported were low, 1% to 7% in mice,<sup>5,6</sup> and 5% to 15% in rats.<sup>10-12</sup> The low transduction was hypothesized to be related to low retroviral titers, which limited the number of viral particles that could be delivered per hepatocyte. Recently, higher retroviral titers have been achieved by using efficient concentration methods or new high titer vector packaging cell lines. Following partial hepatectomy, injections of these high titer vectors in rats resulted in 25%<sup>13</sup> to 46%<sup>14</sup> transduced hepatocytes. Although these results are promising, retroviral-mediated gene transfer to the liver has typically been more efficient in rats than in other species.<sup>2,5,10,15</sup> To avoid methods that induce hepatocellular injury, Bosch et al.<sup>16</sup> used a combination of KGF and highly concentrated retroviral vector to show low efficiency (up to 2%) hepatic gene transfer in mice. Although this was an important step forward, the efficiency of gene transfer was still subtherapeutic for most disorders.

In this study we investigated the use of exogenous rhHGF as a means to achieve retroviral-mediated hepatic gene transfer. Besides being the most potent mitogen for hepatocytes *in vitro*, a number of observations suggest that HGF has potential to induce liver proliferation *in vivo*. Serum levels of HGF rise over 20-fold rapidly after partial hepatectomy and acute toxic liver injury and remain elevated for several days during liver regeneration.<sup>17</sup> Likewise, in humans, HGF serum concentrations rise substantially when the functional hepatic

Abbreviations: KGF, Keratinocyte growth factor; rhHGF recombinant human hepatocyte growth factor; HGF hepatocyte growth factor; NIH, National Institute of Health; IL-6, interleukin-6; BSA, bovine serum albumin,  $\beta$ -Gal, beta-galactosidase; X-gal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside; BrdU, bromodeoxyuridine; TNF, tumor necrosis factor; ALT, alanine amino transferase; mRNA, messenger RNA; IHC, immunohistochemistry.

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mass is decreased,<sup>18</sup> or in liver disease, particularly in fulminant hepatitis.<sup>19</sup> Also, liver regeneration after partial hepatectomy in transgenic mice overexpressing HGF was completed in half the time of normal mice.<sup>20</sup> Nonetheless, in contrast, previous attempts by others to induce liver proliferation by HGF administration in normal intact animals resulted in nondetectable or only low level hepatic DNA synthesis.<sup>21-24</sup> Considering the short intravenous half-life of HGF (<5 minutes) and the substantial sequestration of HGF by extrahepatic organs,<sup>25,26</sup> we hypothesized that high level hepatocyte proliferation may be obtained with continuous infusion of rhHGF directly into the portal circulation and allow for efficient retroviral-mediated hepatic gene transfer.

## MATERIALS AND METHODS

### Animals

Female C57BL/6 and NIH/Bg/Nu/XID (NIH3) mice of 5 to 6 weeks of age were purchased from Taconic Laboratory. IL-6 deficient mice were obtained from Jackson Laboratories. NIH/Bg/Nu/XID (NIH3) mice are deficient in T and B lymphocytes and partially deficient in NK and LAK-cell activity. IL-6 deficient mice display abnormalities in hepatic acute-phase response, some immune mechanisms, and bone resorption in response to estrogen.<sup>27-30</sup> As controls for the IL-6 deficient mice, the B6129F1/J strain (Jackson Labs) of identical age and gender were used.

Animals were housed under specific pathogen-free conditions and treated according to the NIH guidelines for animal care and use, and to the University of Washington Standards. Mice were bled by retroorbital technique and killed by using cervical dislocation. Total body, wet liver, and other major organs were weighed on a XL-3000 digital scale (Fisher, Santa Clara, CA).

### Surgical Procedures

Mice were anesthetized with 0.4 mL of Avertin (20 mg/mL). Portal vein cannulation was as described<sup>31</sup> and the continuous infusion system was described in detail elsewhere.<sup>32</sup> Briefly, the cannula's distal end was tunneled subcutaneously from the abdominal exit site to the posterior scapular region, exteriorized, and connected to a syringe, which was mounted on a multiple syringe pump (Harvard Apparatus, South Natick, MA). To maintain cannula patency, slow infusion of normal sterile saline was started immediately after portal vein cannulation at 0.1 mL/hours. To protect the cannula at the exit site, a mouse tether assembly was attached (Instech Laboratories, Plymouth Meeting, PA). After completion of the retrovirus infusion, the mice were disconnected from the infusion system and the tether was removed under general anesthesia. Sham operated animals underwent identical surgical manipulation as infused mice, except the portal vein cannula was ligated and left in a previously created subcutaneous pocket. Seventy percent partial hepatectomy was performed as described.<sup>5</sup>

### Recombinant Human Hepatocyte Growth Factor

HGF (>90% in active hetero-dimeric form) was produced at Genentech<sup>33</sup> (South San Francisco, CA) and formulated in 20 mmol/L Tris-HCl (pH 7.5) and 500 mmol/L NaCl. Endotoxin levels were <2 endotoxin units/mg, as determined in the limulus assay. HGF was diluted in sterile 0.9% saline, and BSA (1 mg/mL final) (Fisher, Fair Lawn N.J.) was added as a carrier protein.

### Recombinant Retrovirus

The TELCeB6 AF7 packaging cell line, originally described by Cosset et al.<sup>34</sup> (kindly provided by Dr. Takeuchi, London, UK), was used to produce amphotropic retrovirus encoding the nuclear localized *E coli*  $\beta$ -Gal gene. The average retroviral titer was  $0.5 \times 10^8$  colony-forming units/mL (as determined on 208F cells).

The absence of helper virus was reconfirmed.<sup>34</sup> Maintenance of

retroviral packaging cell lines and viral harvest were as described in Kay et al.<sup>5</sup> To ensure a relatively constant retroviral titer during the infusion, a new batch of retrovirus was collected every 8 hours, alternating 2 sets of tissue culture plates. On collection and filtration, rhHGF, 1 mg/mL BSA, and 12  $\mu$ g/mL polybrene (Sigma, St Louis, MO) were added, and the mixture was transferred into a separate syringe for each mouse.

### Recombinant Human Hepatocyte Growth Factor and Retrovirus Administration

Animals were infused continuously with rhHGF diluted in saline (+BSA), saline (+BSA), or with saline alone. In gene transfer studies, rhHGF combined with retrovirus and BSA, or retrovirus alone was infused. The infusion rate of 0.25 mL/hour for 5 days was used for all experiments to allow comparison. The total amount of retrovirus administered over the 5 days was  $1.5 \times 10^9$  cfu. Assuming the normal adult mouse liver has  $10^8$  cells, the total multiplicity of infection was 15.

### Tissue Analysis

**Identification of Proliferating and X-gal Positive Cells.** Mice were injected i.p. with BrdU (30  $\mu$ g/g, Amersham RPN201) 2 hours before they were killed. Livers were fixed in methyl carnoy's fluid for 4 to 6 hours, prepared for histological analysis, and stained by using the Amersham cell proliferation kit. Retrovirus-infused animals were killed at day 5 or 28 after the beginning of the infusion. To detect  $\beta$ -Gal activity, liver tissue was embedded in OCT, snap frozen in methylbutane, and 10 micron sections were fixed with 1.25% glutaraldehyde and stained overnight with X-gal (Sigma, St. Louis, MO). The proportion of BrdU-labeled or X-gal positive nuclei was determined from counting at least 300 cells per  $200 \times$  field from at least 8 random fields. Parenchymal and nonparenchymal cells in liver sections were identified by an experienced liver pathologist. For routine histology, liver samples were fixed in 10% vol/vol formalin, embedded in paraffin, and stained with hematoxylin/eosin.

### Biochemical Measurements

**Tumor Necrosis Factor, Interleukin-6, and Hepatocyte Growth Factor ELISA.** To analyze serum levels of mouse TNF $\alpha$  and IL-6, specific antibodies (20031D, 18071D, 18122D, and 18082D) and cytokine standards (19321T and 19251V) were purchased from Pharmingen (San Diego, CA). The sandwich ELISA was done according to the manufacturer's protocol using TMB tablets to prepare substrate solution (T-3405, Sigma). The linear range of the IL-6 and TNF assay was 150 to 1,200 pg/mL, and 200 to 1,200 pg/mL, respectively. Serum concentrations of recombinant human HGF were measured by using a specific ELISA as reported,<sup>33</sup> by using specific antibodies that were produced against the heterodimer-form of rhHGF. Serum samples were obtained from fresh whole blood (EDTA) and stored immediately at  $-80^\circ\text{C}$  until assayed.

**Alanine Aminotransferase Assay.** As a marker for hepatic injury, serum ALT levels were determined by using a colorimetric diagnostic kit (Sigma, procedure no. 505).

**Serum Biochemistry.** Serum concentrations of cholesterol, triglycerides, total protein, total bilirubin, alkaline phosphatase, and creatinine were measured on a RX Paramax Analyzer (Dade, Irvine, CA).

### RNAse Protection Assay

The mouse TNF $\alpha$ , IL-6, mL32, and mGAPDH templates (mCK-3b, Pharmingen) were used to prepare probes by using the Pharmingen *in vitro* transcription kit. The RPA assay was performed by using the Pharmingen RPA kit. Quantification of the TNF and IL-6 bands was performed on a Model 400S Phosphoimager (Molecular Dynamics, Sunnyvale, CA).

## RESULTS

**Recombinant Human Hepatocyte Growth Factor-Induced Liver Mass Increase**

To determine the biological activity and the most effective amount of intraportal rhHGF for induction of liver proliferation, a dose-response experiment was performed. After placement of an indwelling portal vein catheter (day -1), rhHGF diluted in saline was continuously infused in C57BL/6 mice from days 0 to 5 at 0.25 mL/hour. The largest increase in liver mass relative to total body mass was achieved with a dose of 5 mg/kg/day (Fig. 1A). A higher dose did not further increase

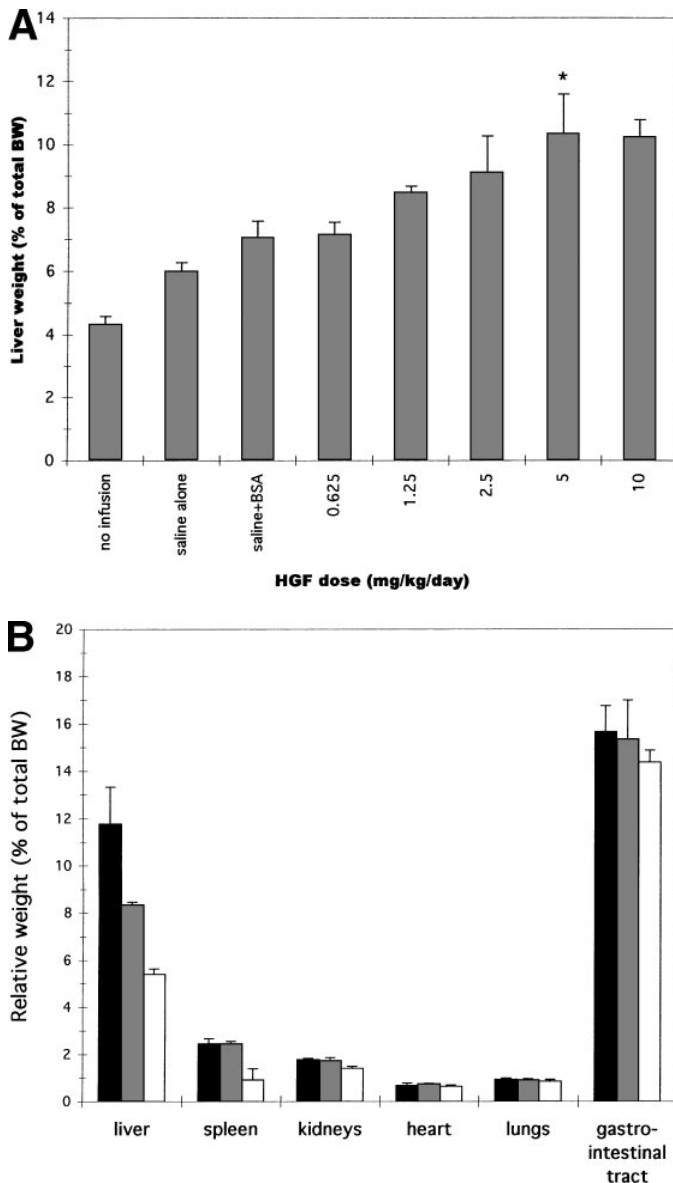


FIG. 1. HGF-induced organ proliferation in mice. (A) Dose-response. Adult C57BL/6 mice were continuously infused into the portal vein with indicated doses of rhHGF for 5 days. Control mice received no infusion, saline alone, or saline plus carrier protein (BSA). On day 5, liver weights were determined as % of total body weight,  $n = 3$  to 9 per group. (B) Organ survey. Mice were continuously infused with HGF (5 mg/kg/day) (solid), or saline plus carrier protein (grey), or saline alone (white). On day 5, animals were sacrificed, and relative organ weights (% of total body weight) were determined,  $n = 3$  to 4 per group. Values represent means and standard deviation. \* $P < .005$  in HGF vs. saline/carrier controls.

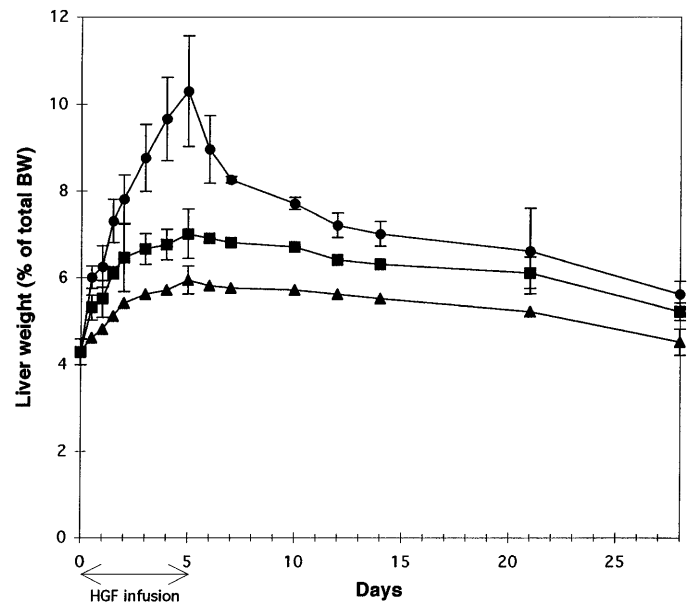


FIG. 2. Time course of HGF-induced liver proliferation. Mice were infused with HGF (5 mg/kg/day) for 5 days (circles). Control mice received saline plus carrier protein (squares), or saline alone (triangles). Mice were killed at indicated times during or after infusion, and relative liver weight was determined (% of total body weight). There were no significant changes in total body weights over the 5 day rhHGF infusion. Values represent means and standard deviation,  $n = 3$  to 9 per time point. Values without error bar represent one animal. At day 5, the largest difference in relative liver weight between HGF infused and saline/carrier controls were observed ( $P < .005$ ).

the relative liver mass. On gross examination at day 5, the livers were strikingly enlarged (140% relative weight increase compared with naive controls and 50% increase compared with carrier controls;  $P < .005$ ), but had otherwise normal morphology. Control animals receiving saline alone or saline and carrier protein showed only moderate but significant liver weight increase (40% and 62%, respectively;  $P < .001$  compared with naive mice). Extrahepatic organs including the kidneys, lungs, and intestines appeared normal and were not enlarged (Fig. 1B). The modest increase in spleen mass was equal in controls and probably represented fluid sequestration or proliferation related to the administration of foreign BSA.

To assess the onset and duration of liver mass changes, the relative liver weight was determined at various times after rhHGF administration. The relative liver weight continually increased up until the last day of infusion, and then gradually involuted to its preinfusion weight over a period of 4 to 5 weeks (Fig. 2).

During rhHGF infusion, peripheral serum levels of rhHGF peaked at 24 hours (at 50 ng/mL) and gradually declined to low levels at day 5, while the infused dose remained constant at 5 mg/kg/day throughout the entire period (data not shown). This may be explained by increased clearance by the enlarging mass over time, or by neutralizing antibodies to rhHGF. The latter is, however, unlikely because serum levels already start to decline at 24 hours, which is probably too early for a humoral immune response.

**Recombinant Human Hepatocyte Growth Factor-Induced Liver Proliferation**

BrdU incorporation detected by immunohistochemistry was used to measure the number of S phase cells at various

times after the start of infusion (Fig. 3 and 4). No BrdU staining ( $<0.1\%$ ) occurred in livers before the start of infusion. In rhHGF infused animals, DNA synthesis in hepatocytes was first noted at 24 hours, peaked at 48 hours with 20% of hepatocyte nuclei staining positive (Fig. 3A, 4A), and returned to near baseline levels on day 5. In contrast, saline or saline/carrier control mice (Fig. 3A, 4B,C) had very few hepatocytes ( $<1\%$ ) in S phase during the infusion. Surprisingly, DNA synthesis in nonparenchymal cells (Fig. 3B) (notably Kupffer, endothelial, and stellate cells) was similar in both rhHGF (Fig. 4A) and saline plus BSA (Fig. 4B) infused animals with a maximum at 48 hours (17%-19%), whereas saline alone had no staining (Fig. 4C). This indicates that the foreign protein infusion into the portal circulation induces nonparenchymal cell proliferation. Both hepatocyte

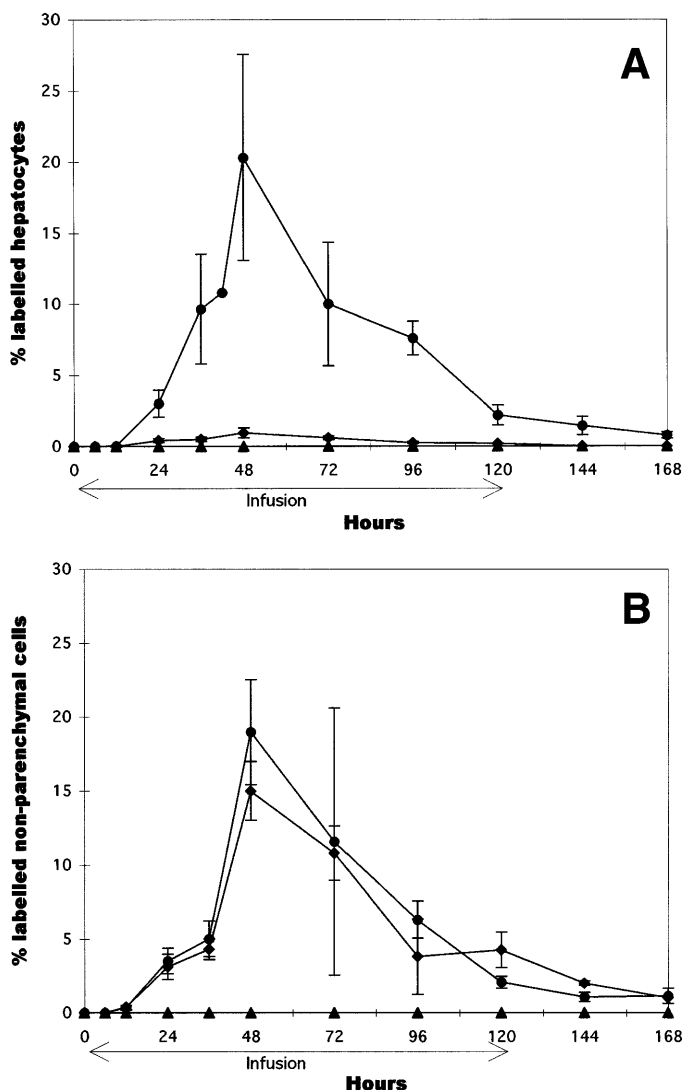


FIG. 3. Hepatic BrdU-incorporation. Mice were infused with HGF (5 mg/kg/day) into the portal vein for 5 days (circles) and injected with BrdU 2 hours before being killed. Control mice received saline plus carrier protein (diamonds), or saline alone (triangles). Mice were killed at indicated times and liver sections were stained by IHC for BrdU. The proportion of stained hepatocyte (A) or nonparenchymal cell (B) nuclei was determined. Values represent means and standard deviation,  $n = 3$  to 4 per time point for HGF infused animals, and  $n = 2$  for saline or saline/carrier control animals.

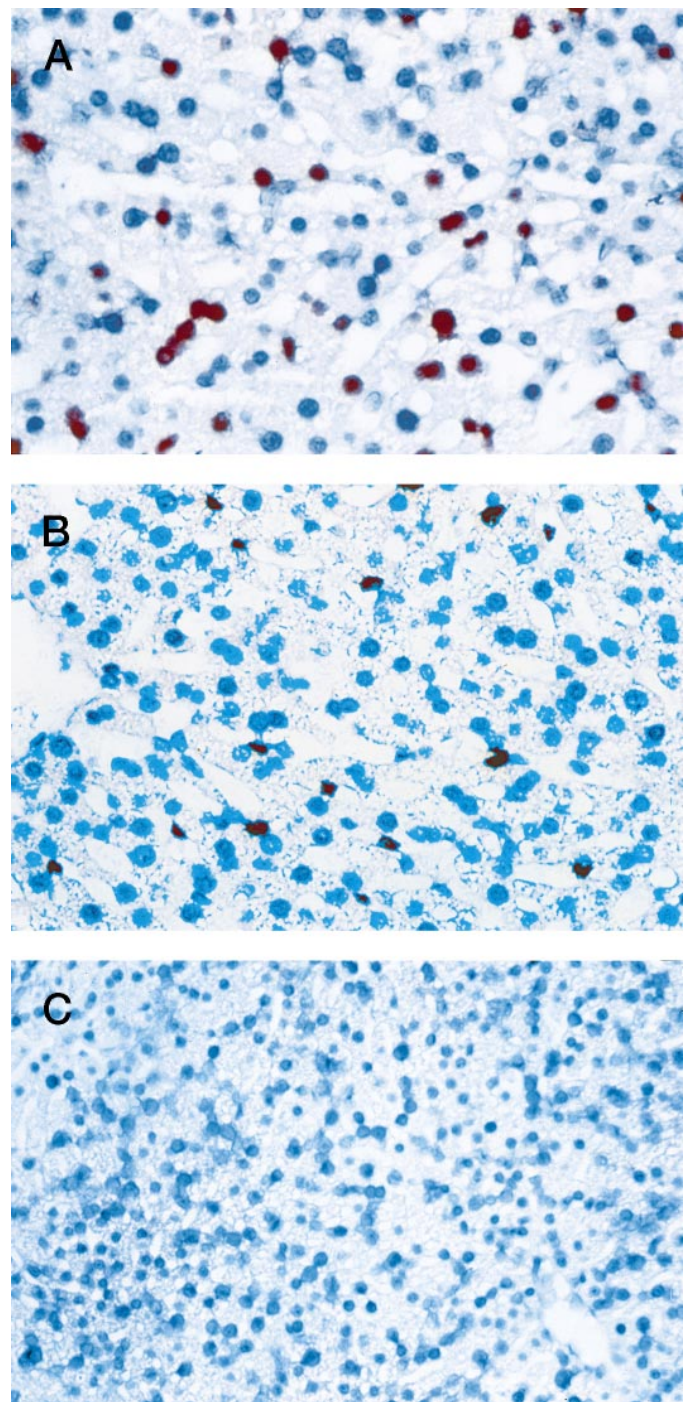


FIG. 4. Immunohistochemical BrdU-labelling. Representative photomicrographs from BrdU-stained liver sections of mice shown in FIG. 3. (A) HGF (5 mg/kg/day) at 48 hours (100 $\times$ ). (B) Saline plus carrier protein at 48 hours (100 $\times$ ). (C) Saline-infused mouse at 48 hours (50 $\times$ ).

and nonparenchymal cell proliferation were equally divided over all three functional zones of the liver in all experimental groups.

Although hepatic hyperplasia was observed, hepatocyte hypertrophy was not detected in rhHGF-treated mice, and hepatocyte density per unit area remained similar to control infused animals (data not shown), suggesting increased cell numbers were responsible for the increased liver weight.

### Recombinant Human Hepatocyte Growth Factor Infusion Allows for Highly Efficient and Persistent Retroviral-Mediated Gene Transfer In Vivo

A high titer retroviral vector expressing  $\beta$ -Gal combined with rhHGF (5 mg/kg/day) was continuously infused for 5 days at 0.25 mL/hour in normal C57BL/6 and in immunodeficient NIH/Bg/Nu/XID mice. At day 5, when relative liver weight was maximal, and at day 28, when the liver had involuted to its original size, mice were sacrificed, and gene transfer efficiency was assayed histochemically by X-gal staining of frozen liver sections (Table 1, Fig. 5). In control mice receiving retrovirus alone without rhHGF, 2.3% (day 5) and 0.33% (day 28) of hepatocytes stained positive. In immunocompetent mice receiving rhHGF, nuclear X-gal staining was positive in 33.6% of the total number of hepatocytes on day 5, and 22.8% on day 28. In immunodeficient mice, 29% were positive on day 5 and 30.5% on day 28. A mild cellular infiltrate was found at day 28 in immunocompetent mice but not in immunodeficient mice.

The transduced cell-types in the rhHGF treated mice were mostly hepatocytes, with occasional nonparenchymal cells. Extrahepatic tissues, including kidney, heart, lung, intestine, brain, skeletal muscle, skin, and gonads did not stain after X-gal histochemistry, while the spleen had very few scattered positive cells ( $<1$  cell/per low power field, data not shown).

#### Safety of Recombinant Human Hepatocyte Growth Factor Infusion

The surgical procedure and infusion of the materials in the study were associated with very low mortality ( $<3\%$ ). All animals appeared healthy and active throughout the infusion and involution without any obvious systemic effects. Besides enlargement, the livers were normal by gross and histological examination on days 1 to 5 during, and day 28 after, rhHGF infusion. To determine whether rhHGF infusion had any adverse effects on liver or kidney function, serum samples were monitored for ALT, a sensitive serum marker for hepatic injury, and for serum total protein, triglycerides, cholesterol, total bilirubin, alkaline phosphatase, and creatinine. The ALT concentrations were slightly elevated during the first 36 hours in animals infused with HGF, saline and BSA, and saline alone, but were within normal range during the remaining infusion period (36-120 hours) (Fig. 6). Sham operated mice also had low level ALT elevation during the first 36 hours postoperatively. Thus, liver injury was not a result of rhHGF, but rather of the operation and/or anesthetic. Serum chemis-

tries were measured daily during HGF infusion and compared with control animals infused with saline plus carrier protein alone ( $n \geq 3$  per time point for each group). The serum cholesterol and total protein concentrations were moderately increased during infusion, but returned to preinfusion levels by day 28 (data not shown). Differences in serum triglycerides, bilirubin, serum alkaline phosphatase, and creatinine concentrations were not significant (data not shown).

#### Recombinant Human Hepatocyte Growth Factor-Induced Liver Proliferation In Vivo Is Independent of the Tumor Necrosis Factor $\alpha$ /Interleukin-6 Pathway

TNF $\alpha$  and IL-6 have recently been shown to be involved in the early events of liver regeneration.<sup>35,36</sup> To investigate whether TNF $\alpha$  and IL-6 are induced by rhHGF-infusion (5 mg/kg/day) serum concentrations of TNF $\alpha$  and IL-6 (Fig. 7A and B), and intrahepatic amounts of the corresponding mRNAs were measured (Fig. 8A and B) during rhHGF infusion. All the experimental and control animals had levels similar to normal mice which were approximately 150 pg/mL for TNF and 200 pg/mL for IL-6. In comparison, after partial hepatectomy, TNF and IL-6 rapidly increased to 900 and 3,200 pg/mL, respectively, and returned to near normal levels by 24 hours. During infusion, hepatic mRNA for TNF was modestly induced in both the rhHGF infused and control groups (Fig. 8A), indicating that this was not a result of rhHGF but rather of the high volume infusion. Induction of IL-6 mRNA was not found at any time during infusion in all experimental groups (Fig. 8B).

To further confirm that the action of rhHGF-induced proliferation was independent of IL-6, HGF was infused in IL-6 knockout mice. rhHGF induced proliferation in IL-6 deficient mice was similar to that seen in normal control mice; after 5 days of rhHGF infusion (5 mg/kg/day), the relative liver weight was 8.9% ( $\pm 0.5$ ) in IL-6 deficient mice versus 9.0% ( $\pm 0.4$ ) in normal mice. This finding was confirmed by BrdU incorporation studies. During the HGF infusion, the number of S phase cells in IL-6 deficient mice was similar to normal mice and peaked at 48 hours after the start of HGF infusion (17%) (data not shown).

## DISCUSSION

In this study, we show that rhHGF continuously infused into the portal vein induces robust hepatocyte proliferation in the normal intact liver in mice, and allows for previously unachievable levels of retroviral-mediated gene transfer *in vivo* (30% of the total number of hepatocytes) without use of methods that induce hepatocellular injury. Even higher transduction efficiencies may potentially be achieved by repeating vector and rhHGF administration after the enlarged liver has returned to its original size, provided that a host immune response against foreign proteins is precluded by using species-specific rhHGF and carrier protein.

Previous attempts by others to induce liver proliferation with use of rhHGF resulted in nondetectable or only low level proliferation, presumably because they used lower doses or otherwise suboptimal experimental conditions.<sup>21-24</sup> Because of the very short intravenous half-life of HGF and the substantial sequestration by extrahepatic organs,<sup>25,26</sup> the continuous and direct supply to the liver likely is a critical factor in delivering biologically effective amounts of HGF to the liver. Although HGF is known to be mitogenic *in vivo* for

TABLE 1. Retrovirus-Mediated Gene Transfer Efficiency

Treatment	Mouse Strain	Blue Cells (%)		Blue Cells (%)	
		Day 5	n	Day 28	n
RV* alone	C57BL/6	2.3 ( $\pm 3.4$ )	6	0.33 ( $\pm 0.6$ )	3
RV* + HGF	C57BL/6	33.6 ( $\pm 7.9$ )	5	22.8 ( $\pm 5.0$ )	4
		( $P < .001$ ) $\dagger$		( $P < .005$ ) $\dagger$	
RV* + HGF	NIH3	29 ( $\pm 5.6$ )	3	30.5 ( $\pm 4.2$ )	4

NOTE. C57BL/6 or NIH3 mice were continuously infused with rhHGF (5 mg/kg/day) and recombinant retrovirus expressing  $\beta$ -Gal ( $0.5 \times 10^8$  cfu/mL) for 5 days at 0.25 mL/hour. Control mice received retrovirus alone. Mice were killed on day 5 or 28 and frozen liver sections were stained with X-gal. The proportion of hepatocyte nuclei staining blue was determined and the values are means  $\pm$  standard deviation.

\*RV = retrovirus.

$\dagger P$  values compare the HGF treated with the RV only group.

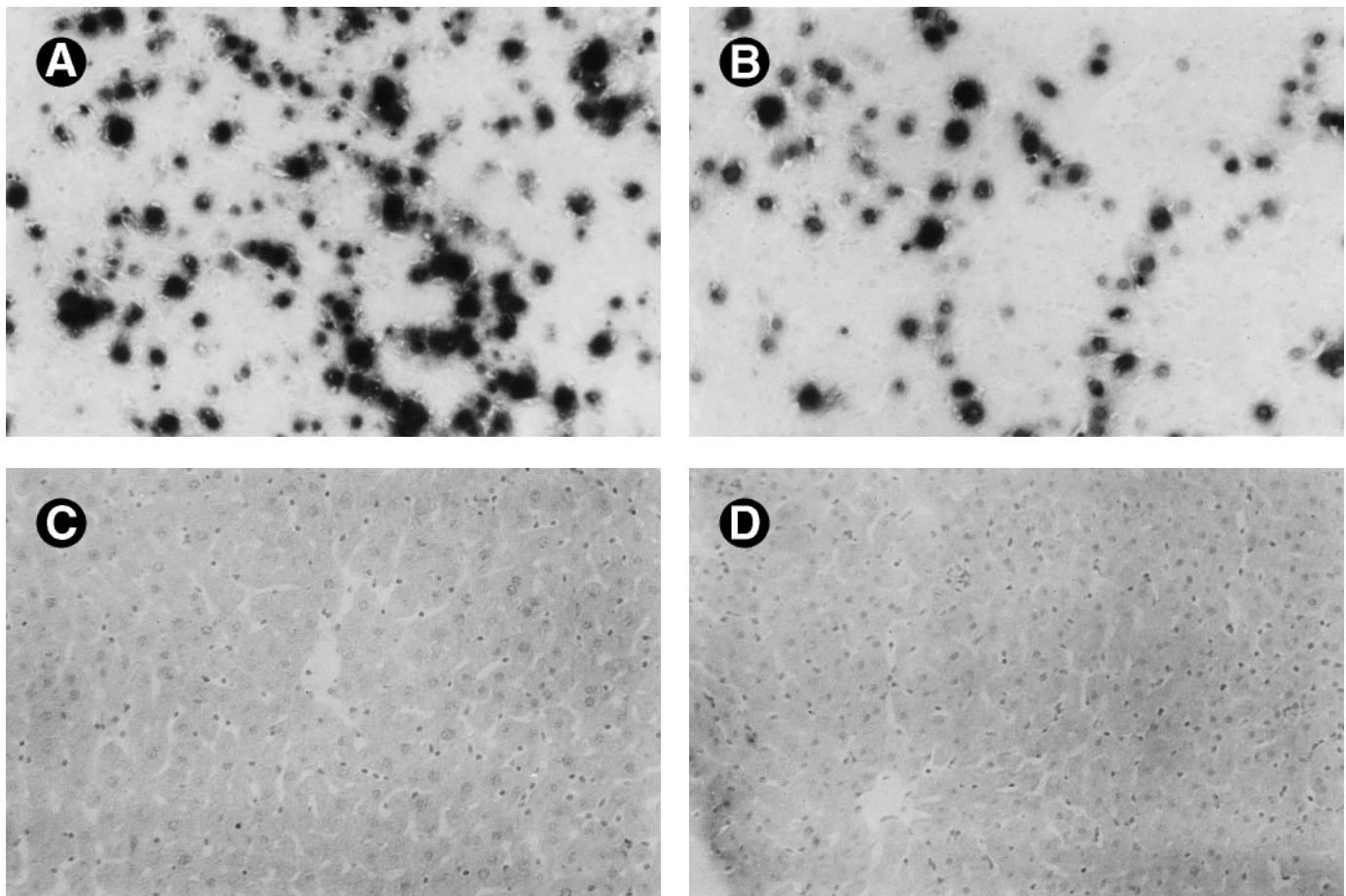


FIG. 5. Hepatic gene transfer efficiency. Representative photomicrographs from X-gal stained liver sections (see Table 1). Infusion of retrovirus and rhHGF in C57BL/6, (A) day 5 (50 $\times$ ), and (B) day 28 (50 $\times$ ). Infusion of retrovirus alone in C57BL/6, (C) day 5 (50 $\times$ ), and (D) day 28 (50 $\times$ ).

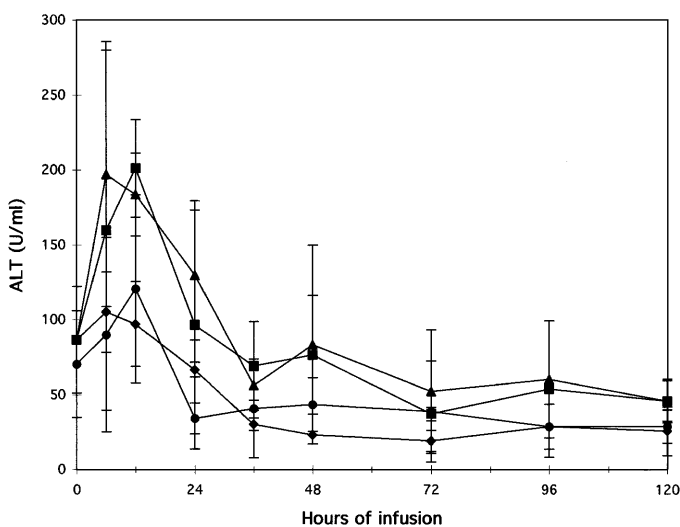


FIG. 6. Serum ALT concentrations. Mice were infused into the portal vein with rhHGF (5 mg/kg/day) for 5 days at 0.25 mL/hour (circles). Control mice received saline plus carrier protein (squares), saline alone (triangles), or were sham operated and received no infusion (diamonds). Serum samples were collected on indicated time points and analyzed for ALT. Values represent means and standard deviation, n = 3 to 9 per time point.

several epithelial tissues other than the liver, in particular, the kidneys,<sup>37</sup> lungs,<sup>38</sup> and intestines,<sup>39</sup> in our study no extrahepatic organs except the spleen were significantly affected or enlarged. It is not possible to exclude low levels of nonhepatic epithelial cell proliferation without more detailed studies. Nevertheless, the route of administration into the portal vein may be critical for targeting HGF specifically to the liver because the liver is the principle clearance organ of HGF with a high first-pass removal ratio.<sup>25,26,40</sup> The enlargement of the spleen was likely a result of proliferation in response to the foreign carrier protein (BSA), because similar splenic enlargement was observed in the carrier control animals, but not when saline alone was infused. Splenic proliferation may be prevented by using species-specific rHGF and carrier protein.

Infusion of saline with carrier protein (without rhHGF) caused some liver weight increase whereas saline alone resulted in only a minor increase in liver weight. The increased liver weight may be caused by infiltrating cells or by osmotic swelling in the liver. However, no histological abnormalities or evidence of infiltration were observed after 5 days of infusion. Interestingly, infusion of saline with BSA induced proliferation of nonparenchymal liver cells, but not hepatocytes, whereas infusion of saline alone did not induce proliferation of either cell type. Apparently, proliferation of nonparenchymal cells in our model is regulated differently than of hepatocytes. This notion is consistent with a recent study showing that regeneration of nonparenchymal cells was

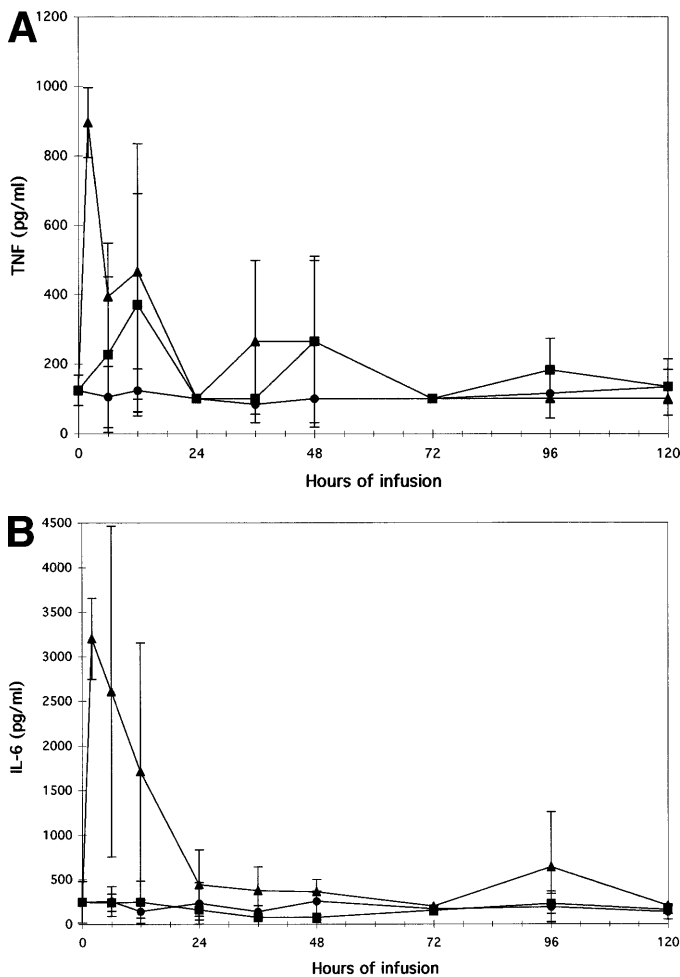


FIG. 7. Serum TNF and IL-6 concentrations. Mice were infused with rhHGF (5 mg/kg/day) for 5 days (circles). Control mice received saline plus carrier protein (squares) or underwent 70% partial hepatectomy (triangles). Serum (A) TNF and (B) IL-6 concentrations were determined at the indicated times. Values represent means and standard deviation,  $n = 3$  to 9 per time point.

normal after partial hepatectomy in IL-6 deficient mice, whereas hepatocyte regeneration was markedly impaired.<sup>35</sup>

As shown in several other models of liver hyperplasia,<sup>41,42</sup> the process of liver involution after rhHGF-induced proliferation is presumably mediated by apoptosis, and ends when the liver mass has returned to its original size. After involution (day 28), the percentage of transduced cells was slightly less (statistical significance was borderline) compared to the final day of rhHGF-infusion (day 5) in immuno-competent mice, but remained the same in immuno-deficient mice. This suggests that the apoptotic process equally affected transduced and nontransduced cells, whereas the slight loss of  $\beta$ -Gal positive cells in immuno-competent mice is consistent with reports that C57BL/6 mice elicit a significant immune response to  $\beta$ -Gal.<sup>43</sup>

To date, recombinant KGF was the only growth factor known to be able to stimulate high level proliferation of hepatocytes in intact liver when injected *in vivo*. In a recent study, recombinant KGF-induced liver proliferation in normal mice allowed for retroviral-mediated gene transfer, but the transduction efficiency was only 1% to 2% of the total number of hepatocytes.<sup>16</sup> There are several possible reasons

for the differences in the results of that study compared to ours. In the recombinant KGF study, only two doses of the protein were administered, the total recombinant KGF dose was relatively low and was not injected into the portal vein. In addition, the total amount of virus administered was  $0.2 \times 10^8$  cfu (in two doses) versus  $1.5 \times 10^9$  cfu in our study. *In vivo*, the transduction rate is however not linear with the

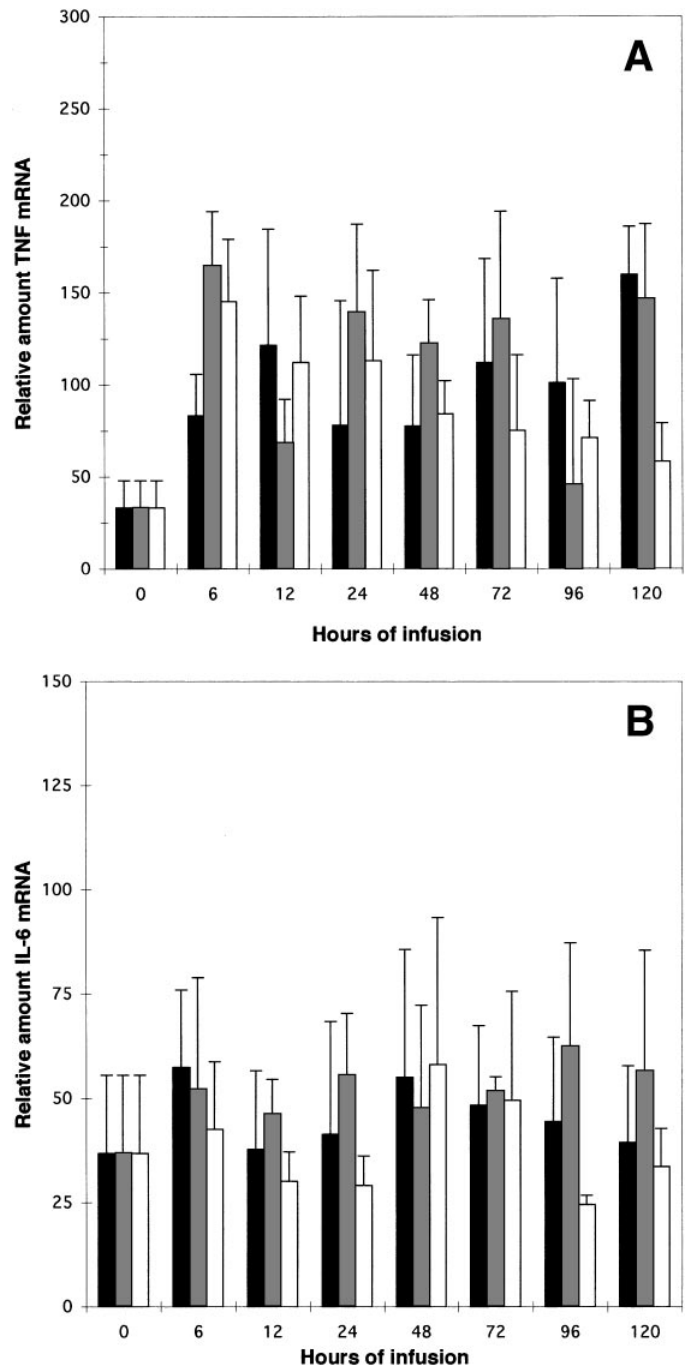


FIG. 8. Hepatic TNF and IL-6 mRNA concentrations. Mice were infused with rhHGF (5 mg/kg/day) for 5 days (solid). Control mice received saline plus carrier protein (grey) or saline alone (white). Mice were killed at indicated times, hepatic mRNA was isolated, and relative amounts of (A) TNF and (B) IL-6 mRNA were determined with a RNase protection assay. Bands were quantitated by phospho-imager and normalized to housekeeping genes L32 and GAPDH mRNA. Values represent means and the standard deviation ( $n = 3$  per time point).

multiplicity of infection and is likely influenced by many factors.<sup>15,44</sup>

In addition to hepatic gene therapy, our findings are of value to liver biology. An important function for HGF in liver regeneration is suggested by many experimental observations,<sup>45</sup> including a rapid and sustained rise in plasma levels after hepatic tissue loss,<sup>17-19</sup> and the ability of HGF to induce expression of a number of transcription factors (C/EBP $\beta$ , STAT3, and NF- $\kappa$ B<sup>46,47</sup>) and immediate early genes (LRF-1 and IGFBP1).<sup>48</sup> The precise role of HGF in the signalling pathways leading to liver regeneration remains however as yet unknown.<sup>45</sup> Although our model using intact animals may not reflect the status after partial hepatectomy, our demonstration that a relatively high dose of rhHGF can induce robust liver proliferation in normal animals supports an important role for HGF in liver regeneration. In our studies, total HGF serum levels (venous, nonportal) achieved during rhHGF infusion were in fact still markedly lower compared to those seen after partial hepatectomy in rats.<sup>17</sup> This may be explained by different ratios of biologically inactive single-chain versus active two-chain HGF. After partial hepatectomy, a large proportion of HGF circulates in single-chain conformation before being converted into the two-chain form when bound to the liver,<sup>25,49</sup> whereas our rhHGF preparation was >90% in the active two-chain form.

Recently, convincing evidence has become available that the TNF $\alpha$ /IL-6/STAT3 signaling pathway must be intact for liver regeneration to proceed normally after partial hepatectomy,<sup>35,36</sup> and IL-6 was implicated as a key factor in the initiation of liver regeneration.<sup>35,50</sup> However, in IL-6 deficient mice nonparenchymal cell regeneration was shown to be similar to normal control mice, and hepatocyte regeneration was eventually, albeit more slowly, completed. This indicates that mitogenic signals other than IL-6 must have reached the liver. In our study we showed that IL-6 is not needed for, or induced by rhHGF-induced liver proliferation in intact animals. HGF-induced proliferation in IL-6 deficient mice was similar to that seen in normal control mice, and serum concentrations of TNF $\alpha$  and IL-6 and intrahepatic amounts of the corresponding mRNAs remained normal during rhHGF infusion. One explanation may be that DNA synthesis is stimulated by entirely different mechanisms in intact versus hepatectomized animals. More likely, these observations suggest that during liver regeneration, IL-6 may either induce HGF production (i.e., IL-6 acts upstream from HGF), and/or IL-6 may potentiate the effect of HGF. This hypothesis is consistent with recent evidence that IL-6 enhances expression of both HGF<sup>51</sup> and the specific HGF-receptor, c-Met.<sup>52</sup>

The stimulation of liver proliferation by rhHGF is relatively safe when compared to conventional methods that induce regeneration. Importantly, there is no functional deficit of the liver at any time during liver proliferation or involution. In addition, the liver fully retains its normal macro- and micro-architecture. As reported by others,<sup>22,23</sup> serum cholesterol and total protein were slightly elevated during HGF infusion, but returned to normal when the liver mass had involuted to preinfusion level. The slight elevation in ALT during the first 36 hours was similar in saline-infused and sham-operated control animals, indicating that this was not caused by rhHGF itself but may be caused by the operation or anesthesia. Transgenic mice highly over-expressing HGF were reported to eventually develop tumors, which was strongly linked to chronic autocrine activation and simulta-

neous overexpression of both HGF as well as the c-Met receptor.<sup>53,54</sup> For gene therapy purposes, only a short course of exogenous rhHGF infusion is needed, and this would not necessarily pose a risk of tumorigenicity. In fact, HGF was found to suppress the growth of hepatocellular carcinoma *in vivo*.<sup>55,56</sup> Clearly, studies in larger animals will be needed to formally establish the safety of short course HGF administration. Ultimately, liver-specific promoters can be used as an additional safety feature for organ-specific gene expression. For clinical application, short term access to the portal circulation for vector and rhHGF administration may be obtained safely by using minimally invasive techniques. Alternatively, HGF and vector may be administered into the hepatic artery, which can be safely accessed by femoral artery catheterization.

In conclusion, we present evidence that exogenous rhHGF is a powerful mitogen for the mature intact liver in mice. Our findings are encouraging in that rhHGF induced proliferation represents a relatively safe system that can be used to achieve persistent and efficient retroviral-mediated gene transfer. By using retroviral vectors of similar titer encoding therapeutic proteins, therapeutic levels of systemic gene expression for genetic liver diseases such as hemophilia may be achieved by this method. In addition, we show that the HGF-induced proliferation was independent of IL-6, an essential cytokine involved in liver regeneration after partial hepatectomy.

Finally, the evidence that rhHGF is a potent hepatocyte mitogen *in vivo* may have another clinical implication. Direct attempts to stimulate liver regeneration in humans have thus far been few and largely unsuccessful. Studies in rodents show that, aside from its enhancing effects on liver regeneration following liver injuries,<sup>23,24</sup> exogenous rhHGF also suppressed the onset of liver cirrhosis by dimethylnitrosamine<sup>57-59</sup> and decreased morbidity and mortality in animal models of hepatotoxicity and cholestasis.<sup>60-62</sup> In addition, HGF has recently been shown to prevent liver failure caused by endotoxemia in rats.<sup>63</sup> These and our present findings suggest that rhHGF, in addition to providing a powerful method for hepatic gene therapy, may have potential in the clinical treatment of liver failure.

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