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Intramuscular Injection of an Adenoviral Vector Expressing Hepatocyte Growth Factor Facilitates Hepatic Transduction with a Retroviral Vector in Mice

CUIHUA GAO, RODNEY JOKERST, PRATHIMA GONDIPALLI, SHI-RONG CAI, SUSAN KENNEDY, and KATHERINE PARKER PONDER

ABSTRACT

Retroviral vectors can result in therapeutic and stable levels of expression of proteins from the liver. However, most retroviral vectors transduce only dividing cells, and hepatocytes are normally quiescent. The goal of this study was to determine if an adenoviral vector could transiently express hepatocyte growth factor (HGF) in order to induce hepatocyte replication and facilitate retroviral vector transduction of the liver. Intramuscular injection of an adenoviral vector that expressed human HGF from the cytomegalovirus promoter (Ad.CMV.HGF) resulted in moderate levels of HGF in blood and liver, and replication of 3 to 12% of hepatocytes. No cytopathic effect was observed in the liver, and a control adenoviral vector induced no or lower levels of replication. When a retroviral vector expressing β-galactosidase cDNA was injected into a peripheral vein during the peak period of hepatocyte replication induced by intramuscularly administered Ad.CMV.HGF, 8% of hepatocytes were transduced. We conclude that intramuscular injection of Ad.CMV.HGF is a safe and effective way to induce transient systemic expression of HGF and hepatocyte replication, and to facilitate transduction of hepatocytes with a retroviral vector.

OVERVIEW SUMMARY

Retroviral vectors can result in stable and therapeutic levels of expression of proteins from the liver. However, most retroviral vectors transduce the liver *in vivo* only when the normally quiescent hepatocytes are induced to replicate. Although 70% partial hepatectomy, 70% portal branch occlusion, or administration of a liver toxin have been used to induce hepatocyte replication for gene therapy, their potential morbidity makes them less attractive for use in humans. Growth factors that induce hepatocyte replication are a promising approach for facilitating retroviral vector transduction. However, they are expensive, and optimal results require continuous infusion or multiple injections. We demonstrate here that intramuscular injection of Ad.CMV.HGF resulted in the transient appearance of HGF in the serum and liver, hepatocyte replication, and retroviral vector transduction without apparent toxicity. This approach will be a useful tool for performing gene therapy experiments in large numbers of animals with a specific genetic disorder.

INTRODUCTION

HEPATIC GENE THERAPY could permanently correct genetic deficiencies by transferring a functional gene into hepatocytes (Ponder, 1997). Plasmid DNA generally results in low-level transient expression in the liver (Perales *et al.*, 1994). Adenoviral vectors result in a high level of expression that is usually transient owing to the immune rejection of cells that express residual adenoviral genes (Kay *et al.*, 1994; Connelly *et al.*, 1996; Wang *et al.*, 1997; Kung *et al.*, 1998), although long-term expression has been observed in some rodents without immunosuppression. Adeno-associated virus (AAV) vectors have resulted in both high-level and long-term expression from the liver in mice (Snyder *et al.*, 1997; Nakai *et al.*, 1998), but peak expression is not observed until 2–3 months after delivery and high numbers of viral particles are required.

Retroviral vectors can be made to deliver genes to hepatocytes *in vivo* by using a closed perfusion system (Ferry *et al.*, 1991) or by injecting them into the portal vein (Kay *et al.*, 1992; Rettinger *et al.*, 1994) or a peripheral vein (Bosch *et al.*, 1996; 1998). We have demonstrated that this approach results in ther-

Departments of Internal Medicine and Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO 63110.

apeutic levels of the coagulation protein factor X (Le *et al.*, 1997) or the anticoagulation protein C (Cai *et al.*, 1998) for more than 1 year in rats. However, the major drawback to the *in vivo* delivery of retroviral vectors is the need to induce hepatocyte replication for efficient gene transfer, as Moloney murine leukemia virus (Mo-MuLV)-based vectors transduce only dividing cells (Miller *et al.*, 1990), and hepatocytes are normally quiescent. Although human immunodeficiency virus (HIV)-based vectors can transfer genes into nondividing liver cells, the safety of these vectors remains a concern, and the efficiency appears to be low (Kafri *et al.*, 1997).

Most investigators have used a 70% partial hepatectomy (PH) to induce the hepatocyte replication that is necessary for *in vivo* transduction with Mo-MuLV retroviral vectors (Kay *et al.*, 1992; Rettinger *et al.*, 1994). The need to perform a major surgical procedure is a strong impediment to using *in vivo* delivery of these vectors in humans. Partial portal branch occlusion induces apoptosis of the region of the liver that is occluded and compensatory replication of the nonoccluded liver in both rats (Bowling *et al.*, 1996) and pigs (Duncan *et al.*, 1999) with little morbidity. However, this percutaneous procedure still could have adverse clinical effects in some patients.

A safe and effective nonsurgical method for inducing hepatocyte replication would increase the feasibility of using Mo-MuLV-based vectors for gene therapy. Kaleko *et al.* (1991) achieved retroviral vector transduction after induction of liver cell replication by CCl_4. Lieber *et al.* (1995a) used an adenoviral vector that encoded the toxic urokinase plasminogen activator (uPA) gene to obtain a high labeling index and transduction of hepatocytes *in vivo*. Lower toxicity was observed when the uPA gene was modified to prevent it from being secreted (Lieber *et al.*, 1995b; Patijn *et al.*, 1998a). Although toxins can induce liver cell replication and allow retroviral transduction to occur, the response might be difficult to control, and could result in some deaths due to liver failure.

An alternative approach to inducing hepatocyte replication is to use hepatic growth factors. Hepatocyte growth factor (HGF), transforming growth factor α (TGF-α), keratinocyte growth factor (KGF), insulin, and glucagon can induce or augment hepatocyte replication *in vitro* (Fausto *et al.*, 1995; Michalopoulos and DeFrances, 1997). Furthermore, hepatocyte replication has been stimulated *in vivo* in rodents by the administration of HGF (Ishika *et al.*, 1992; Fujiwara *et al.*, 1993; Liu *et al.*, 1994; Webber *et al.*, 1994; Ishii *et al.*, 1995; Roos *et al.*, 1995), TGF-α (Liu *et al.*, 1994; Webber *et al.*, 1994), KGF (Housley *et al.*, 1994), or triiodothyronine (T_3; Forbes *et al.*, 1998). In addition, HGF has augmented replication after 30% partial hepatectomy (Kobayashi *et al.*, 1996) or partial portal branch occlusion (Ueno *et al.*, 1996) in dogs. Finally HGF, KGF, or T_3 have facilitated retroviral vector transduction *in vivo* in rodents. However, the percentage of retroviral vector-transduced cells was low with peripheral administration of HGF (Bosch *et al.*, 1998; Kosai *et al.*, 1998), KGF (Bosch *et al.*, 1996), both HGF and KGF (Bosch *et al.*, 1998), or T_3 (Forbes *et al.*, 1998). A higher labeling index was achieved by continuous portal vein (PV) infusion of HGF for 5 days, but this approach required specialized expertise and a dose of HGF that is not readily available to most investigators (Patijn *et al.*, 1998b).

The purpose of this study was to determine if an adenoviral vector could transiently express sufficient levels of HGF *in vivo* to induce high-level hepatocyte replication and facilitate retroviral vector transduction *in vivo*. We report here that intramuscular injection of an adenoviral vector that expresses HGF facilitates *in vivo* retroviral vector transduction of the liver. We conclude that intramuscular injection of this adenoviral vector will be an effective tool for testing the ability of retroviral vectors to have a clinical effect in the livers of animals with genetic disorders.

MATERIALS AND METHODS

Generation of recombinant adenoviral vector

Plasmid pSPORT-hHGF, carrying the 2.3-kb human HGF cDNA (Zarneger *et al.*, 1991) whose protein product can stimulate replication of human or rodent hepatocytes, was obtained from G. Michalopoulos (University of Pittsburgh, Pittsburgh, PA). pACCMV.pLpA was obtained from R. Gerard (University of Michigan, Ann Arbor, MI) (Becker *et al.*, 1994). The HGF cDNA was cloned downstream of the cytomegalovirus (CMV) promoter of pACCMV.pLpA as a *Bam*HI fragment to generate pACCMV.HGF. This plasmid was cotransfected into 293 cells (American Type Culture Collection, Rockville, MD) with Ad.RR5, an E1/E3-deleted adenoviral vector (Alcorn *et al.*, 1993) that was derived from pJM17 (Graham and Prevec, 1991), and cells were overlaid with agar (Becker *et al.*, 1994). Homologous recombination generates an adenoviral vector designated Ad.CMV.HGF that contains most of the Ad5 sequences and the HGF gene. Plaques were picked, used to infect 293 cells on a 24-well plate, and the conditioned medium was tested in the HGF enzyme-linked immunosorbent assay (ELISA) described below. Three rounds of plaque purification were performed. The purified adenoviral vector was shown to be free of wild-type virus by infecting HeLa cells and observing the absence of plaques, and by performing the polymerase chain reaction (PCR) using E1A-specific primers and observing the absence of a signal (Easton *et al.*, 1998).

Preparation of adenoviral vectors

For large-scale adenoviral preparation, 293-N3S cells (Microbix Biosystems, Toronto, Ontario, Canada) were grown in suspension in Joklik-modified minimal essential medium with Earle's salts and glutamine (GIBCO-BRL, Grand Island, NY) with 10% horse serum (Sigma, St. Louis, MO) to a density of 5×10^5 cells/ml. The cells were infected with adenoviral vector at a multiplicity of infection (MOI) of 10. The cells were pelleted at 40–44 hr after infection by centrifugation at $800 \times g$ for 10 min at 4°C, resuspended in 10 m*M* $NaPO_4$ pH 6.8, subjected to three cycles of freezing in a dry ice–ethanol bath followed by thawing at 37°C, and the adenoviral particles were purified on a $CsCl_2$ gradient as described (Becker *et al.*, 1994). Adenoviral particles were dialyzed against 10 m*M* Tris-HCl (pH 7.4)–1 m*M* $MgCl_2$, sterilized by passage through a 0.45-μm pore size filter, and stored in 10% glycerol at 4°C until use. Adenoviral titer was determined by measuring the optical density (OD) at 260 nm (1 OD = 10^{12} particles/ml; Becker *et al.*, 1994).

Human growth factor ELISA

A goat anti-human HGF IgG antibody (AB-294-NA) was purchased from R&D Systems (Minneapolis, MN). This antibody was conjugated to horseradish peroxidase (HRP) using a kit from Pierce (Rockford, IL). The ELISA was based on the procedure of Harlow and Lane (1988). ELISA plate wells were incubated with 100 μl of uncoupled antibody diluted 1:200 in 0.1 *M* $NaHCO_3$, pH 9.5, at 37°C for 2 hr. The plates were rinsed three times with 200 μl of TBS (0.1 *M* NaCl–0.05 *M* Tris, pH 7.5). Two hundred microliters of TBS–milk (TBS with 5% fat-free Carnation [Nestle, Glendale, CA] dry milk and 0.02% [g/dl] sodium azide) was added per well and the plate was refrigerated overnight or longer. Just prior to assay, the plate was rinsed three times with TBS. One hundred microliters of each sample diluted in TBS–milk was added per well and incubated at 37°C for 2 hr. The wells were rinsed six times with 200 μl of TBS with 0.05% Tween 20 (TBS–Tween). The HRP-coupled anti-human HGF antibody was diluted 1:200 in TBS–milk and 100 μl was added per well for 2 hr at room temperature. The wells were rinsed six times with TBS–Tween. One hundred microliters containing 3,3′,5,5′-tetramethylbenzidine dihydrate (0.1 mg/ml), 24 m*M* citrate, 52 m*M* Na_2HPO_4, and 0.06% H_2O_2 was added and the reaction was stopped after 3–5 min by adding 100 μl of 2 *N* sulfuric acid per well. The optical density was read in an ELISA plate reader at 450 nm. HGF standards were generated using single-chain HGF obtained from Becton Dickinson Labware (Bedford, MA). Samples were diluted if necessary to give an optical density that was within the linear portion of the standard curve.

HGF biological activity

HGF was tested for its ability to scatter Madin–Darby canine kidney (MDCK) cells as described (Rosen *et al*., 1990) with slight modifications. MDCK cells (50,000) were plated on a 12-well plate (2.2-cm-diameter wells) in modified Eagle's medium (MEM) with 10% supplemented calf serum (CS) for 1 day to allow clusters of cells to form. One hour before adding HGF, the medium was changed to MEM with 5% CS. HGF was diluted serially in MEM with 5% CS and incubated with the cells for 24 hr. The concentration of HGF that induced 50% scattering was determined.

Determination of HGF levels in liver

Approximately 100 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 m*M* $CaCl_2$, 0.01% Tween 80, 1 m*M* phenylmethylsulfonyl fluoride, pH 8.5) as described previously (Asami *et al.*, 1991). The homogenate was centrifuged in a microcentrifuge at 12,000 × *g* for 15 min and the supernatant was assayed by ELISA to determine HGF levels. Total protein levels were determined using a Bradford assay kit obtained from Bio-Rad (Hercules, CA).

Animal procedures

Female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) that were 4 to 6 weeks old received standard institutional care. For intramuscular injection of adenoviral vector, mice were anesthetized with inhaled metofane (Mallinckrodt Veterinary, Mundelein, IL), and varying doses of purified adenoviral vectors in a total volume of 300 μl were injected into the tibialis anterior (50 μl per leg) and the quadriceps muscle (100 μl per leg) on both sides, using a 30-gauge needle. For bromodeoxyuridine (BrdU) staining, either one or three doses of BrdU (100 mg/kg per dose) were injected intraperitoneally. The animal was sacrificed 2 hr after the last dose of BrdU, and the liver was frozen in optimal cutting temperature (O.C.T.) compound (Bayer, Mishiwaka, IN). For retroviral vector transduction, 300 μl of concentrated retroviral vector was injected into the tail veins of mice twice a day on days 3, 4, and 5 after injection of adenoviral vectors or phosphate-buffered saline (PBS) into muscle. Blood samples were collected from the retroorbital plexus in microhematocrit capillary tubes and serum was assayed for HGF by ELISA.

BrdU labeling

BrdU immunostaining was performed on 8-μm frozen sections as described in detail (Bowling *et al*., 1996) and sections were counterstained with eosin. The labeling index was determined by counting the total number of BrdU-labeled hepatocytes and nonparenchymal (NP) cells in 20 different high-power (×40) fields that were chosen randomly. This was divided by the average total number of hepatocytes (161, or 70% of all cells) or NP cells (70, or 30% of all cells) observed in the same size field in a normal liver that was processed in a similar fashion but was stained with hematoxylin and eosin (H&E).

Preparation of retroviral vector and X-Gal staining

The high-titer amphotropic retroviral vector, designated TA7, that expresses *Escherichia coli* β-galactosidase (β-Gal) with a nuclear localization signal (Cosset *et al*., 1995) was generously provided by F. Cosset (Ecole Normale Supérieure de Lyon, Lyon, France). Before injection into animals, thirty 15-cm-diameter plates were incubated with 12 ml of fresh Dulbecco's modified Eagle's medium (DMEM) per plate for 12 to 16 hr, with high glucose (GIBCO-BRL) and 2% CS. The conditioned medium was ultrafiltered with an M14S-260-01N ultrafiltration device with a molecular mass cutoff of 400 kDa (Spectrum, Laguna Hills, CA) for approximately 1 hr, resulting in a 100-fold decrease in volume and a 10-fold increase in retroviral vector titer to an average titer of 2×10^8 blue-forming units/ml on NIH 3T3 cells using a modification of the procedure of Bowles *et al.* (1996). Cells and liver sections were staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as reported previously (Ponder *et al*., 1991).

RESULTS

Construction of Ad.CMV.HGF and in vitro *analysis*

The recombinant adenoviral vector designated Ad.CMV.HGF is shown in Fig. 1. Conditioned medium from 293 cells that were infected for 48 hr with Ad.CMV.HGF at an MOI of 10 contained HGF at 0.5 to 2 μg/ml as determined by ELISA. Western blot after electrophoresis on a reducing sodium dodecyl sul-

FIG. 1. Adenoviral vector Ad.CMV.HGF. The recombinant adenoviral vector Ad.CMV.HGF contains the CMV promoter (CMV), the 2.3-kb human HGF cDNA (HGF), and the intron (I) and polyadenylation site (A) from SV40 in an E1/E3-deleted Ad5 backbone.

fate (SDS)-polyacrylamide gel demonstrated that most of the HGF was cleaved into the biologically active form with two chains of 69 and 34 kDa (data not shown). This HGF had a level of biological activity in a scatter assay performed on MDCK cells similar to that of recombinant HGF purchased from a commercial source (data not shown). Ad.RR5 (Alcorn *et al.*, 1993) is an E1/E3-deleted adenoviral vector that contains the CMV promoter without a downstream gene, and is otherwise identical to Ad.CMV.HGF. Conditioned medium from Ad.RR5-infected 293 cells had no detectable HGF by ELISA or Western blot, and had no scatter activity (data not shown).

BrdU staining after intramuscular injection of adenoviral vectors

Previous studies have demonstrated that intramuscular injection of adenoviral vector results in localized expression in the muscle and systemic expression of secreted proteins (Kung *et al.*, 1998). Initial studies involved the injection of Ad.CMV.HGF or the control adenoviral vector into muscle in an attempt to express HGF systemically and induce hepatocyte replication *in vivo*. The effect of the adenoviral vectors on hepatocyte replication was determined by measuring the percentage of liver cells that incorporated BrdU into their nuclei at various times thereafter. BrdU is a thymidine analog that is incorporated only into cells that are synthesizing DNA.

Figure 2 demonstrates representative examples of BrdU staining, while Fig. 3 shows quantitation of the percentage of replicating hepatocytes (Fig. 3A) and NP cells (Fig. 3B). Control mice that did not receive any adenoviral vector had few replicating cells, as shown in Fig. 2A. In contrast, intramuscular injection of 2.5×10^{12} particles of Ad.CMV.HGF into a 4-week-old mouse resulted in replication of 12.4% of hepatocytes at 4 days (Fig. 3A). Replicating cells were scattered randomly throughout the hepatic lobule (Fig. 2B and C). This dose of Ad.CMV.HGF resulted in average replication rates of 5 to 12% of hepatocytes 3–5 days after intramuscular injection into 4-week-old mice (Fig. 3A), which was significantly higher than the level of 1.6% observed in normal 4-week-old mice. Hepatocyte replication had fallen to normal values by 7 days after injection. Replicating hepatocytes were present at somewhat lower levels (Fig. 2D; 7.2% in Fig. 3A) 4 days after 3×10^{12} particles of Ad.CMV.HGF were injected intramuscularly into a 6-week-old mouse. For this group, the average rate of replication of 3 to 5% of hepatocytes at 2–5 days was significantly higher than the labeling index of 0.2% observed in normal 6-week-old mice.

Although these data demonstrate that intramuscular injection of Ad.CMV.HGF results in hepatocyte replication, it was possible that this was due to nonspecific effects of the adenoviral vector, and not to the expression of HGF. We therefore tested if injection of control adenoviral vectors would have the same effect. The number of BrdU-labeled hepatocytes on day 4 (Fig. 2E; 4.4% in Fig. 3A) was much lower after injection of 3×10^{12} particles of the control adenoviral vector Ad.RR5 than after injection of a similar number of particles of Ad.CMV.HGF into a 4-week-old mouse. The value at 4 days, but not at other time points, was significantly higher than the labeling index observed in normal 4-week-old mice, but was 2.5-fold lower that the level observed in mice that received Ad.CMV.HGF. Intramuscular injection of 3×10^{12} particles of Ad.RR5 into a 6-week-old mouse resulted in few labeled hepatocytes (Fig. 2F; 0.1% in Fig. 3A) on day 4. For a number of animals, this dose of Ad.RR5 resulted in replication of $\leq 1.5\%$ of hepatocytes at 2 to 6 days in 6-week-old mice, which was higher than, but not significantly different from, the labeling index in control mice that did not receive any adenoviral vector. We conclude that Ad.CMV.HGF results in much higher levels of hepatocyte replication in either 4- or 6-week-old mice than in control mice of the same age that receive no adenoviral vector. The control adenoviral vector appeared to result in a modest increase in hepatocyte replication.

Some increase in the labeling index was also observed in NP cells of the liver after intramuscular injection of adenoviral vectors, as quantitated in Fig. 3B. Labeled NP cells were most prominent for the 4-week-old mice that received Ad.RR5 (Fig. 2E), and can be identified by the small size of the nuclei and the fact that the cytoplasm does not stain with eosin. Both vectors resulted in replication of 1 to 7% of all NP cells 3 to 5 days after intramuscular injection into 6-week-old animals. For both vectors, the peak labeling index observed 5 days after injection was significantly different from the value of 0.6% in normal mice. For 4-week-old animals, the control vector Ad.RR5 induced higher levels of replication of NP cells (up to 20%) than did Ad.CMV.HGF (4%) for reasons that were not clear. We conclude that induction of replication of NP cells is probably a nonspecific effect of intramuscular administration of an adenoviral vector.

The histology of the livers was normal after injection of 3×10^{12} particles of Ad.CMV.HGF or the control Ad.RR5 vector. The normal liver histology correlated well with the absence of toxicity of the vector, as no deaths or adverse effects have been noted after intramuscular injection of 3×10^{12} particles of Ad.CMV.HGF into more than 35 mice.

Quantitation of serum and liver HGF levels

Determination of HGF levels in blood and liver are critical for understanding the relative efficacy of this approach. Serum HGF levels were 0.3 to 2 ng/ml on days 1 to 5 in mice that received an intramuscular injection of 2.5 to 3×10^{12} particles of Ad.CMV.HGF, regardless of the age of the animal (Fig. 3C).

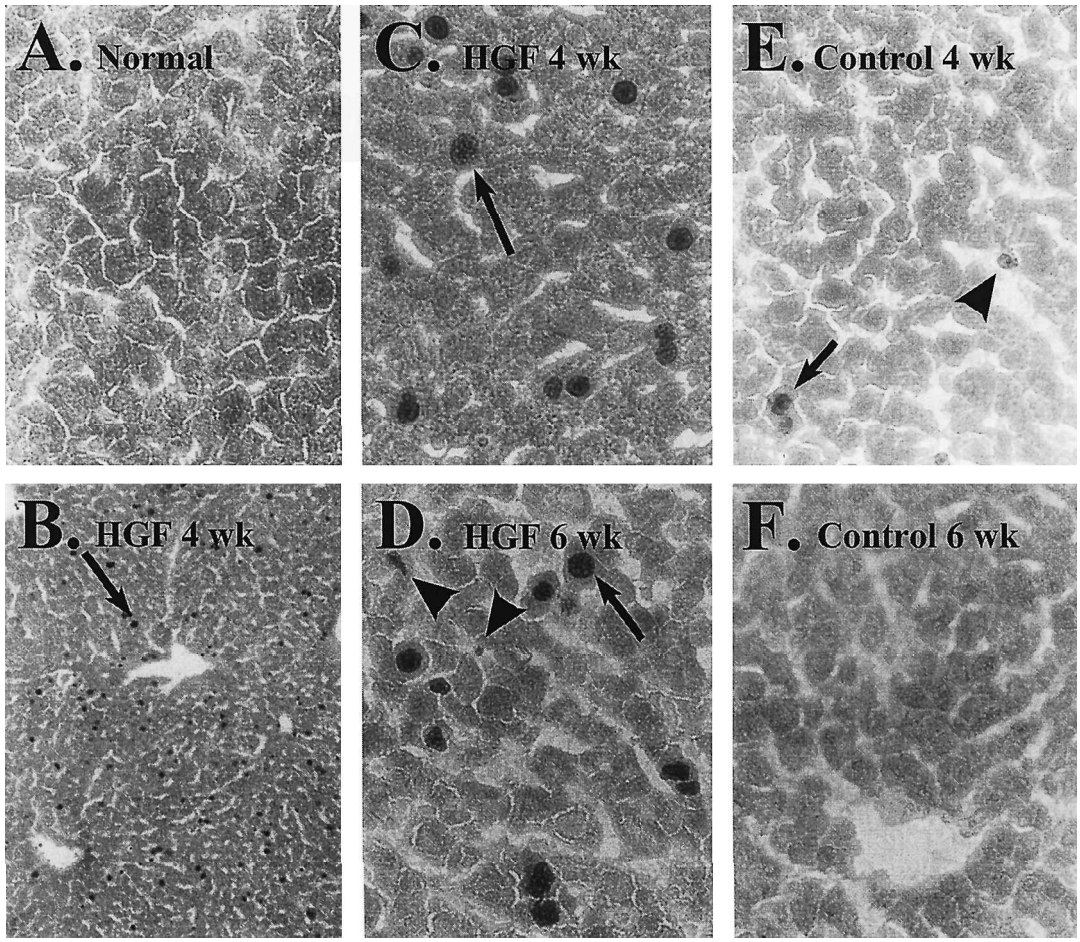

FIG. 2. BrdU labeling after injection of adenoviral vectors into muscle. Mice received an intramuscular injection of Ad.CMV.HGF or a control adenoviral vector. Three to 5 days later, they were injected once, intraperitoneally, with BrdU (100 mg/kg) 2 hr prior to sacrifice. Immunocytochemistry was performed on frozen liver sections using an anti-BrdU antibody. All sections were counterstained with eosin. **(A)** Normal liver. A liver from a 6-week-old normal mouse that did not receive any adenoviral vector, but did receive BrdU, had few labeled cells. (**B** and **C**) Ad.CMV.HGF (2.5×10^{12} particles injected intramuscularly) in a 4-week-old mouse. This animal was sacrificed 4 days after the intramuscular injection of Ad.CMV.HGF, at which point 12.4% of the hepatocytes were replicating. Labeled cells are located randomly throughout the hepatic lobule. Arrows indicate BrdU-labeled hepatocytes. **(D)** Ad.CMV.HGF (3×10^{12} particles injected intramuscularly) in a 6-week-old mouse. This animal was sacrificed 4 days after the intramuscular injection of Ad.CMV.HGF, at which point 7.2% of the hepatocytes were replicating. Arrowheads indicate BrdU-labeled nonparenchymal cells, 4.2% of which were replicating. **(E)** Control adenoviral vector Ad.RR5 (3×10^{12} particles injected intramuscularly) in a 4-week-old mouse. This animal was sacrificed 4 days after the intramuscular injection of Ad.RR5, at which point 4.4% of hepatocytes were replicating. **(F)** Ad.RR5 (3×10^{12} particles) in a 6-week-old mouse. This animal was sacrificed 4 days after intramuscular injection of Ad.RR5, at which point 0.1% of hepatocytes and 0.6% of nonparenchymal cells were replicating. Original magnification: **(A)** ×40; **(B)** ×10; **(C)** ×40; **(D)** ×40; **(E)** ×40; **(F)** ×40.

Liver HGF levels were 0.5 to 7 ng of HGF per milligram total protein 3 to 9 days after injection in these animals, but had fallen to lower levels by day 10, as shown in Fig. 3D. Both serum and liver HGF levels were significantly higher at many of the time points after intramuscular injection of Ad.CMV.HGF compared with the levels observed in normal mice that did not receive any adenoviral vector. Both serum and liver HGF levels were low in mice that received 3×10^{12} particles of the control vector Ad.RR5, and none of the values were significantly different from the levels observed in normal mice.

Dose response to intramuscular injection of adenoviral vectors

Although the preceding data suggested that intramuscular injection of Ad.CMV.HGF was an effective way to induce hepatocyte replication, the optimal dose was not clear. We therefore performed an additional experiment to optimize the intramuscular dose of Ad.CMV.HGF for inducing hepatocyte replication. Figure 4A demonstrates that a threefold higher intramuscular dose (6×10^{12} particles) than that used in the pre-

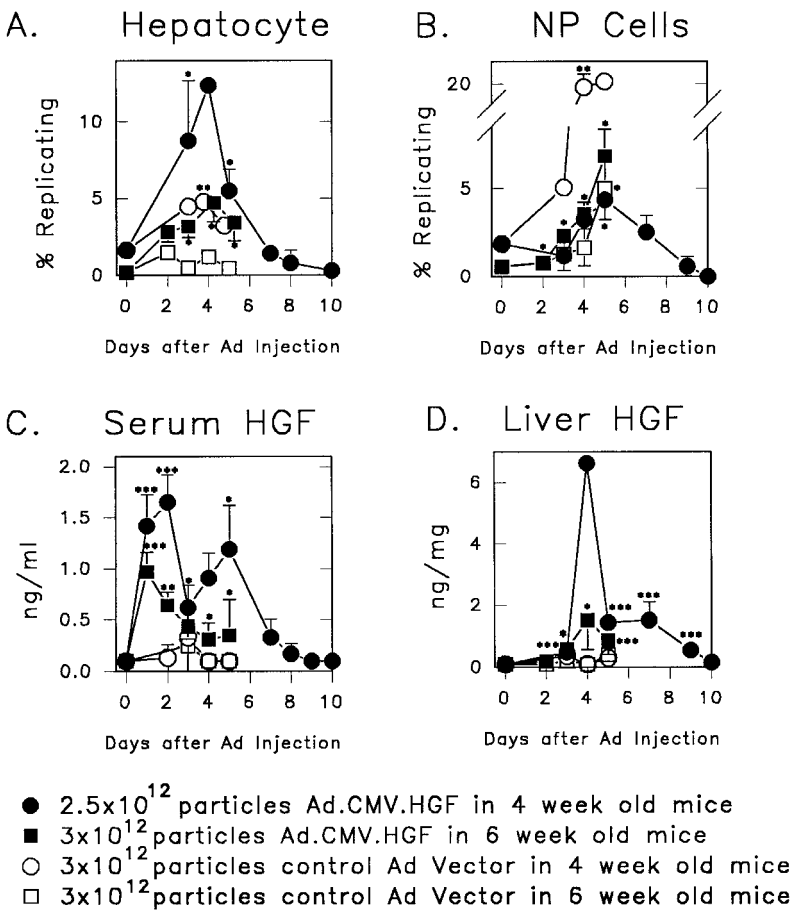

FIG. 3. Quantitation of the labeling index in the liver, HGF levels in serum, and HGF levels in liver after intramuscular administration of Ad.CMV.HGF or a control adenoviral vector. Mice that were 4 or 6 weeks old received an intramuscular injection of Ad.CMV.HGF or the control adenoviral vector Ad.RR5, as indicated in the legend. **(A)** Hepatocyte labeling index. Animals were sacrificed at various times after the injection of adenoviral vector, and BrdU staining was performed as described in Materials and Methods and in the caption to Fig. 2. The percentage of all hepatocytes that were labeled with BrdU was determined. For this and all other panels, averages ± standard error of the mean (SEM) are shown. Values were compared with those determined in normal mice, using the Student *t* test. $*p = 0.05$ to 0.005; $**p = 0.005$ to 0.0005; $***p < 0.0005$. The value in age-matched normal controls is shown as the zero time point for each line. **(B)** Nonparenchymal cell labeling index. The percentage of all nonparenchymal cells that were labeled with BrdU was determined. **(C)** Serum HGF levels. Serum was collected at various times after the injection of adenoviral vector, and HGF levels determined by ELISA. Normal mice had serum HGF levels of 0.02 ng/ml, which is the value shown at the zero time point. **(D)** Liver HGF levels. HGF levels in a liver extract were determined and normalized to total protein. Normal mice had liver HGF levels of 0.03 ng of HGF per milligram of total protein, which is the value shown at the zero time points.

ceding experiment resulted in high serum HGF levels of 50 to 150 ng/ml 1–4 days after injection. Figure 4B demonstrates that liver HGF levels 4 days after injection was also high at 242 ng of HGF per milligram protein. After intramuscular injection of 2×10^{12} particles of Ad.CMV.HGF, both serum and liver HGF on days 1 and 4 were somewhat higher than was observed in the previous experiment. This difference was probably due to variation in the preparation of adenoviral vector, as a different batch was used here than was used in the previous experiment. Injection of 6×10^{11} particles of Ad.CMV.HGF resulted in undetectable levels of HGF in serum and low levels of HGF in liver.

The labeling index determined for this dose escalation study is shown in Fig. 4C. These numbers cannot be directly compared with those shown in Fig. 3, as these animals received three total doses of BrdU over a 24-hr period, while animals in earlier experiments received a single dose of BrdU. Three percent of hepatocytes were replicating on days 3 and 4 after injection of 6×10^{11} particles of Ad.CMV.HGF, which was only twofold higher than the percentage of labeled hepatocytes in normal animals that received three doses of BrdU in the same fashion. Injection of 2×10^{12} and 6×10^{12} particles of Ad.CMV.HGF resulted in replication of 10 and 12%, respectively, of hepatocytes during this time interval. We conclude that although intramuscular injection of 6×10^{12} particles of Ad.CMV.HGF results in higher levels of HGF in serum and liver than does 2×10^{12} particles, the levels of hepatocyte replication 3–4 days after injection are only marginally higher.

Hepatomegaly is a potential adverse effect of administration of hepatic growth factors. We therefore measured the percent-

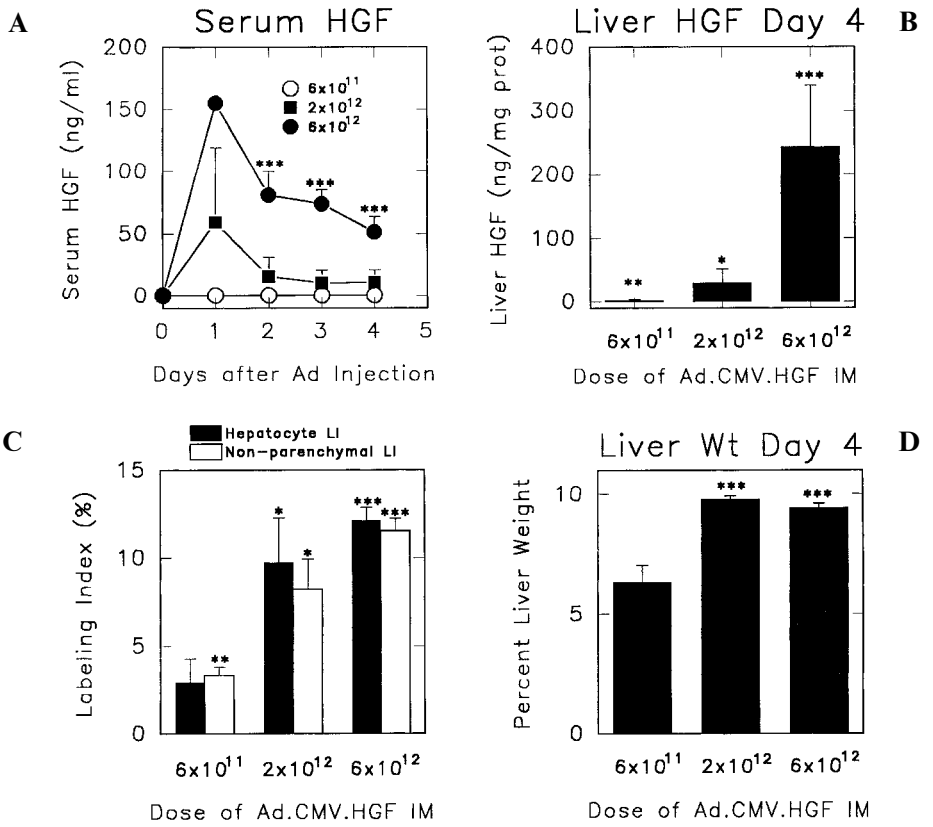

FIG. 4. Effect of increasing the dose of Ad.CMV.HGF on serum and liver HGF levels, replication in the liver, and liver weight. Six-week-old mice were injected intramuscularly, with 0.6×10^{12}, 2×10^{12}, or 6×10^{12} particles of Ad.CMV.HGF. For **(A–D),** averages ± SEM are shown. Values were compared with those determined in normal mice, using the Student *t* test. $*p = 0.05$ to 0.005; $**p = 0.005$ to 0.0005; $***p < 0.005$. **(A)** Serum HGF levels. Serum was tested for HGF levels by ELISA. **(B)** Liver HGF levels. Animals were sacrificed 4 days after injection of Ad.CMV.HGF, and HGF levels in a liver extract determined and normalized to total protein levels. **(C)** BrdU labeling index. Mice were injected with BrdU at 72, 84, and 96 hr after the injection of Ad.CMV.HGF, and sacrificed at 98 hr. Immunostaining for BrdU was performed on frozen liver sections and the percentage of labeled hepatocytes and NP cells determined. **(D)** Liver weight. The liver weight was determined at the time of sacrifice, 4 days after injection of Ad.CMV.HGF, and divided by the total body weight.

age of the body weight that was occupied by the liver at the time of sacrifice (4 days after injection) in this study. The low dose (6×10^{11} particles) of Ad.CMV.HGF did not induce hepatomegaly, which is consistent with the fact that the HGF levels and labeling index were low. In contrast, injection of the medium dose (2×10^{12} particles) or the high dose (6×10^{12} particles) resulted in a twofold increase in liver size by 4 days. This hepatomegaly had resolved 2 weeks or later after injection of 2×10^{12} particles of Ad.CMV.HGF (data not shown). However, some animals that received the higher dose of 6×10^{12} particles died spontaneously within 2 weeks after injection and were noted to have marked hepatomegaly at autopsy. We conclude that the higher dose resulted in more prolonged hepatomegaly and toxicity.

Retroviral vector transduction

The ultimate goal of this study was to use transient expression of HGF to facilitate retroviral vector transduction. We therefore tested if intramuscular injection of the medium dose (2×10^{12} particles) of Ad.CMV.HGF would facilitate retroviral vector transduction. The retroviral vector TA7 used in this study expresses the β-Gal protein with a nuclear localization signal from the long terminal repeat promoter of the retroviral vector, which results in blue staining in the nucleus of transduced cells. Normal mice that were 5–6 weeks old were injected intramuscularly with a single dose of PBS, 2×10^{12} particles of the control adenoviral vector Ad.RR5, or 2×10^{12} particles of Ad.CMV.HGF. Serum HGF levels 3 days after the intramuscular injection were 0, 0, and 11.2 ± 2.4 ng/ml, for mice that received PBS, Ad.RR5, or Ad.CMV.HGF, respectively. This confirmed that Ad.CMV.HGF resulted in expression of HGF in this group of animals. The retroviral vector TA7 was then injected via the tail vein twice a day on day 3, day 4, and day 5, which was the period during which hepatocyte replication was at its peak in previous studies.

Livers were obtained 1 week after the last injection of retroviral vector, and X-Gal staining performed on frozen sections as shown in Fig. 5 and quantitated in Fig. 6. Only 0.14 ± 0.09% of hepatocytes were transduced with the retroviral vector in mice that received PBS (Fig. 5A). Mice that received Ad.RR5 prior to retroviral vector had transduction of 1.6 ± 1% of he-

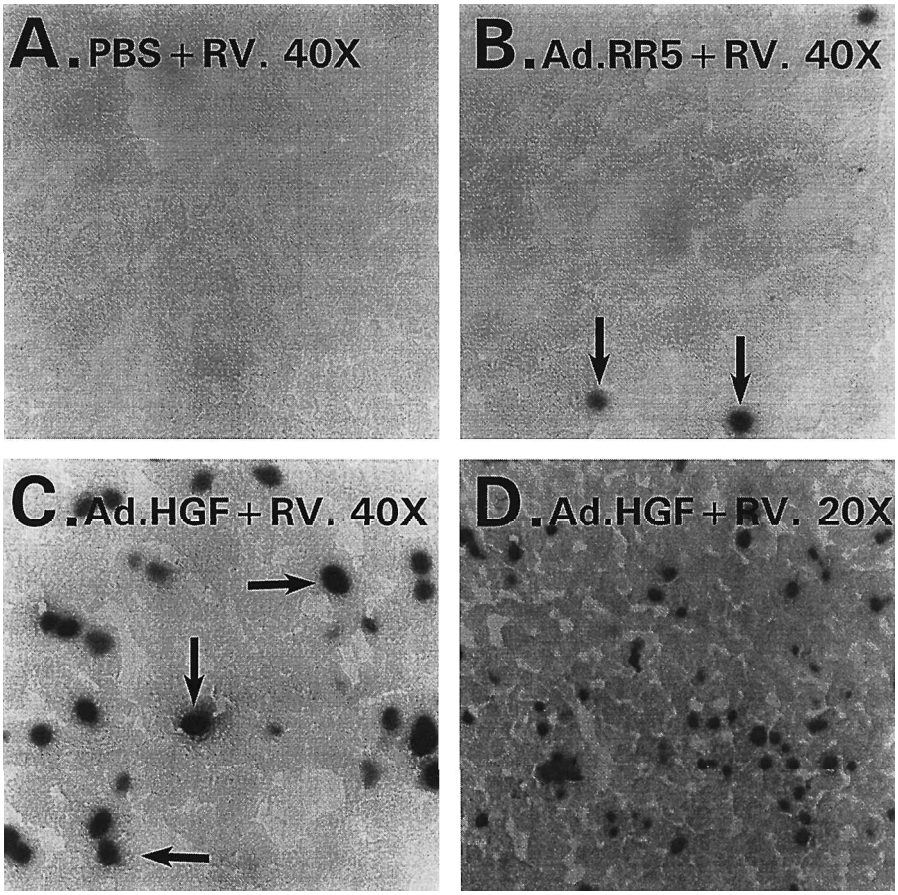

FIG. 5. Histochemical staining for β-Gal activity after transduction with TA7. PBS, 2×10^{12} particles of Ad.RR5, or 2×10^{12} particles of Ad.CMV.HGF were injected into the muscles of 5- to 6-week-old mice. Retroviral vector was injected twice on days 3, 4, and 5 via the tail vein, for a total dose of 3.6×10^{8} blue-forming units. Since a normal mouse liver contains about 2×10^{8} hepatocytes, this represents an MOI of $\sim$2. One week later, mice were sacrificed and liver sections were stained for β-Gal activity. **(A)** Mouse that received PBS and TA7: No transduced cells were present in this field. **(B)** Mouse that received Ad.RR5 and TA7: There are occasional transduced hepatocytes, which are identified by black arrows. (**C** and **D**) Mouse that received Ad.CMV.HGF and TA7: Large numbers of labeled hepatocytes are identified. No labeled parenchymal cells were present. Original magnification: **(A–C)** $\times$40; **(D)** $\times$20.

patocytes (Fig. 5B), which was 11-fold higher that the number observed in mice that received PBS, although the differences were not significant owing to marked variation in the transduction efficiency for the mice that received Ad.RR5. Mice that received Ad.CMV.HGF had transduction of 7.9% of hepatocytes (Fig. 5C and D), which was 55-fold ($p = 0.014$) and 5-fold ($p = 0.016$) higher than the number of transduced cells from mice that received PBS or Ad.RR5, respectively. We conclude that Ad.CMV.HGF results in a marked increase in the number of hepatocytes that can be transduced with a retroviral vector over that observed with PBS alone, and a modest increase over that observed with a control adenoviral vector. Although BrdU staining had demonstrated that up to 7% of NP cells replicated in mice of this age that received either Ad.RR5 or Ad.CMV.HGF, few or no transduced NP cells were identified.

DISCUSSION

Transduction of hepatocytes with standard Mo-MuLV-based retroviral vectors has been successful, but has been hampered by the need to induce hepatocyte replication by surgery or toxins for efficient gene transfer. A simple and safe method for inducing hepatocyte replication would greatly increase the feasibility of using these vectors for gene therapy. We demonstrate here that intramuscular injection of an adenoviral vector is an effective way to induce transient hepatocyte replication and to facilitate retroviral vector transduction in animals.

Intramuscular injection of Ad.CMV.HGF induces hepatocyte replication

In this study, intramuscular injection of 2×10^{12} particles of Ad.CMV.HGF resulted in replication of 3 to 5%, and 5 to 12%, of hepatocytes on day 3, 4, or 5 after delivery into 6- and 4-week-old animals, respectively. The levels of replication were significantly higher than the levels observed in age-matched normal mice or mice that received a control adenoviral vector that did not encode HGF. The higher replication rate in the younger animals may have been due to increased responsiveness of a young liver to hepatic growth factors. It has previously been shown that a treatment that induced low levels of

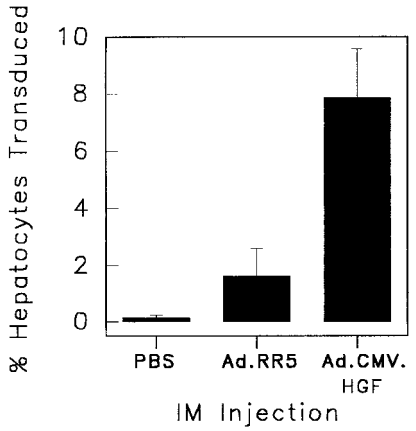

FIG. 6. Quantitation of the percentage of transduced cells. The average percentage of hepatocytes that were transduced after TA7 retroviral vector injection was determined for PBS-treated mice ($N = 3$), Ad.RR5-treated mice ($N = 4$), and Ad.CMV.HGF-treated mice ($N = 5$). Averages ± SEM are shown.

hepatocyte replication, such as 30% PH, could prime the liver to respond more effectively to administration of either HGF or TGF-α (Webber *et al.*, 1994). Since livers from younger mice have a higher baseline replication rate than do livers from older mice, hepatocytes from young animals might be more responsive to hepatic growth factors. Alternatively, the slightly higher levels of HGF that were achieved in the younger animals may have led to the higher replication rate. These results suggest that younger animals may be more amenable to growth factor-facilitated retroviral vector transduction because they are more responsive to the effects of HGF, although studies using purified protein will need to be done to confirm this.

Unexpectedly, the control adenoviral vector Ad.RR5 appeared to have a modest effect on hepatocyte replication at most time points between 3 and 5 days after intramuscular injection, although the levels were significantly different from normal mice only at 4 days in the 4-week-old mice. We have preliminary data that a dose of endotoxin that induces the acute-phase response in rats induces low levels of hepatocyte replication in the liver (Gao and Ponder, 1999). Furthermore, others have demonstrated that intravenous injection of adenoviral vectors induces the acute-phase response (Lieber *et al.*, 1997; Loser *et al.*, 1998). We therefore believe that intramuscular injection of adenoviral vectors may have induced a sufficient acute-phase response to induce low levels of replication of hepatocytes in the liver, although we have no data that directly support this hypothesis.

Intramuscular injection of Ad.CMV.HGF induces low levels of replication of nonparenchymal cells

Both Ad.CMV.HGF and the control adenoviral vector Ad.RR5 induced replication of NP cells in the liver, which was an unexpected finding. We believe that this effect may be due to the induction of the acute-phase response by intramuscular injection of adenoviral vector. We have preliminary data that endotoxin, an inducer of the acute-phase response, induces replication of NP cells in the liver. As discussed already, intravenous injection of adenoviral vector can induce the acute-phase response (Lieber *et al.*, 1997; Loser *et al.*, 1998), raising the possibility that intramuscular injection might have a similar effect.

Serum and liver HGF levels after intramuscular injection of Ad.CMV.HGF

The serum levels of HGF that induced replication of hepatocytes *in vivo* after intramuscular injection of Ad.CMV.HGF were relatively low at 0.5–2 ng/ml. This level is higher than the serum HGF levels of 0.24 ng/ml previously reported in normal humans (Tsubouchi *et al.*, 1991), and higher than the level of 0.02 ng/ml that we detected in normal mice using an anti-human-HGF antibody, which might underestimate the true values owing to lack of cross-reactivity with mouse HGF. The serum HGF levels in these animals were lower than the level of human HGF, 5–10 ng/ml, that induces maximal replication of rat and human hepatocytes *in vitro* (Strain *et al.*, 1991), and lower than the level of 8 ng/ml observed in human patients with fulminant hepatic failure (Tsubouchi *et al.*, 1991). However, serum HGF levels may not be an accurate measurement of the delivery of HGF to the liver, as HGF binds avidly to glycosaminoglycans in the liver, and it is known that 30% of HGF localizes to the liver within 15 min after intravenous injection (Appasamy *et al.*, 1993).

Retroviral vector transduction after intramuscular injection of Ad.CMV.HGF

In this study, intramuscular injection of Ad.CMV.HGF followed by tail vein injection of TA7 increased the number of hepatocytes that were transduced to 55-fold relative to that in mice that received PBS and retroviral vector, and to 5-fold relative to that in mice that received the control adenoviral vector Ad.RR5 and retroviral vector, resulted in an overall transduction efficiency of 7.9% of hepatocytes. This level of transduction should result in a plasma level sufficient to correct the clinical manifestations of many genetic disorders such as hemophilia (Le *et al.*, 1997). The control adenoviral vector appeared to have a modest effect on transduction of hepatocytes, although these values were not significantly different from the levels achieved after intramuscular injection of PBS owing to marked variation in the animals that received Ad.RR5. Increased hepatocyte transduction after injection of Ad.RR5 likely reflects its modest induction of hepatocyte replication, as already discussed. Although low numbers of replicating NP cells were observed in mice that received both adenoviral vectors, we failed to observe expression of the reporter gene in NP cells after transduction with the retroviral vector TA7. This might reflect a true absence of transduction, or a failure to express the long terminal repeat (LTR) promoter in transduced NP cells.

Others have used injection of hepatic growth factors to induce hepatocyte replication and facilitate retroviral vector transduction. In most studies, systemic injection of KGF, HGF, or T_3 resulted in replication of 5 to 13% of hepatocytes, and transduction of 1 to 2% of hepatocytes. The higher transduction efficiency (7.9%) achieved in this study is likely due to the high titer of retroviral vector particles that were injected over a 3-day period, as our percentage of replicating cells at any one time was comparable to those obtained in most of these studies that used T_3 or growth factor proteins. Our approach was

less efficient than continuous portal vein injection of extremely high doses of HGF and retroviral vector over a 5-day period, in which up to 30% of hepatocytes were transduced. However, our approach did not involve an invasive surgical procedure and amounts of HGF that are not available to most investigators.

Implications for hepatic gene therapy

Although a single intramuscular injection of an adenoviral vector resulted in transient replication of hepatocytes and efficient retroviral vector transduction of hepatocytes in mice in this study, it is unlikely that this approach would be used as gene therapy in humans, for several reasons. First, administration of continuous intravenous or portal vein infusion of HGF protein is simple to perform in humans, although more difficult in small animals. Second, adenoviral vectors have inherent risks, such as recombination to generate wild-type virus, that should be avoided if possible. Third, the injection of an adenoviral vector that contains a growth factor gene that is known to induce tumors in the liver of transgenic mice (Sakata *et al.*, 1996; Takayama *et al.*, 1997) might lead to malignant transformation should a cell integrate the HGF gene into its genome. Although it is unlikely that this approach will ultimately be used in gene therapy for humans, it should prove valuable for facilitating retroviral vector-mediated gene therapy in animals in order to demonstrate a beneficial effect of gene therapy in various models of disease, for two reasons. First, HGF protein is extremely expensive and is not readily available to most investigators. Second, administration of multiple doses of growth factor, implantation of an osmotic pump, or continuous infusion of growth factor appears to be necessary to achieve a high level of hepatocyte replication. All of these procedures are time consuming and require specialized expertise to perform, particularly in small animals. A single intramuscular injection of Ad.HGF.CMV is simple to perform, making studies in large numbers of small and large animals with a specific genetic disorder feasible. In addition, the intramuscular injection of an adenoviral vector expressing HGF should also be useful for determining if there are long-term adverse effects of HGF on the liver or other organs *in vivo*.

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Address reprint requests to:
Dr. Katherine Parker Ponder
Department of Internal Medicine
660 S. Euclid Avenue, Box 8125
St. Louis, MO 63110

E-mail: kponder@imgate.wustl.edu

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