

Increased Hepatic Transduction with Reduced Systemic Dissemination and Proinflammatory Cytokines Following Hydrodynamic Injection of Helper-Dependent Adenoviral Vectors

Nicola Brunetti-Pierri,¹ Donna J. Palmer,¹ Viraj Mane,¹ Milton Finegold,² Arthur L. Beaudet,¹ and Philip Ng^{1,*}

¹Department of Molecular and Human Genetics and ²Department of Pathology, Baylor College of Medicine, Houston, TX 77030, USA

*To whom correspondence and reprint requests should be addressed at the Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, T619, Houston, TX 77030, USA. Fax: +1 713 798 7773. E-mail: png@bcm.tmc.edu.

Available online 8 April 2005

Hydrodynamic injection of helper-dependent adenoviral vectors (HDAd) in mice results in increased hepatic transduction, reduced splenic and pulmonary transduction, and reduced levels of the proinflammatory cytokines IL-6 and IL-12 compared to conventional injection. These results indicate that hepatic transduction by HDAd, at least alone, does not necessarily provoke a severe innate inflammatory response. Instead, they suggest that systemic vector dissemination may play a major role in the severity of the innate inflammatory response. These results further suggest that the safety and efficacy of HDAd-mediated, liver-directed gene therapy may be improved if the vector could be preferentially, if not exclusively, targeted to liver.

Key Words: helper-dependent adenoviral vectors, gutless, adenovirus, hydrodynamic, gene therapy, liver, hepatocytes, kupffer cells, cytokines, adenovirus

INTRODUCTION

The liver is an important target for gene therapy because hepatocytes can secrete therapeutic proteins into the circulation for systemic delivery and because it is the affected organ in many genetic disorders. Helper-dependent adenoviral vectors (HDAd), which are devoid of all viral coding sequences, have demonstrated tremendous potential for liver-directed gene therapy because they can transduce hepatocytes following peripheral intravenous injection to direct long-term transgene expression in the absence of chronic toxicity (reviewed in [1,2]). However, there exists a barrier to efficient hepatocyte transduction following systemic administration of Ad-based vectors such that only at high doses is efficient hepatic transduction observed. Specifically, numerous studies have shown a nonlinear dose response, with low doses yielding very low to undetectable levels of transgene expression, but with higher doses resulting in disproportionately higher levels of transgene expression in mice [3–6] and non-human primates [7–9]. Unfortunately, systemic administration of Ad-based vectors, including HDAd, at the high doses required to achieve efficient hepatic trans-

duction leads to acute toxicity. Consistently, it has been shown in mice and nonhuman primates that low vector doses result in minimal, if any, acute toxicity, while high doses, required for efficient hepatocyte transduction, can lead to severe and lethal acute toxicity [8–10]. This acute toxic response occurs immediately following vector administration, is independent of vector-derived gene expression, and is consistent with viral capsid-mediated activation of an innate inflammatory response, the severity of which is dose-dependent [4,10–12].

That both the efficiency of hepatic transduction and the severity of the acute toxic response are dose-dependent suggests a possible relationship that must be investigated because it has a direct implication on the clinical usefulness of HDAd for liver-directed gene therapy. For example, if high-efficiency hepatic transduction necessarily results in severe acute toxicity, then the utility of HDAd for liver-directed gene therapy must be reevaluated. In this study, we demonstrate that hydrodynamic injection of HDAd results in preferential hepatic transduction and we have used this approach to investigate the important relationship between hepatic transduc-

tion, systemic vector dissemination, and the severity of the innate inflammatory response.

RESULTS

The size of the hepatic sinusoidal fenestrations (normally ≤ 100 nm) is enlarged as a consequence of the high intrahepatic vascular pressures generated by hydrodynamic injection [13]. Because the size of the Ad virion is ~ 100 nm, we hypothesized that hydrodynamic delivery would increase hepatic transduction efficiency.

Hepatic Vector Uptake

To determine if hydrodynamic injection of HDAd would affect the hepatic transduction efficiency, we injected mice under conventional and hydrodynamic conditions with various doses of HD $\Delta 28E4$ LacZ [14]. Forty-eight hours postinjection, we harvested the livers for analyses.

Conventional injection of 1×10^{12} and 5×10^{12} vp/kg yielded qualitatively fewer β -galactosidase-positive cells (Figs. 1A and 1C) than hydrodynamic injection (Figs. 1B and 1D) as determined by X-gal histochemistry. At a dose of 1×10^{13} vp/kg, qualitative differences in transduction efficiency were not distinguishable between hydrodynamic and conventional injection because all cells appeared β -galactosidase positive as determined by X-gal histochemistry, likely because this high dose was saturating (Figs. 1E and 1F). To quantitate the amount of transgene expression, we extracted total protein from the livers and determined the amount of β -galactosidase activity. The results revealed that hydrodynamic injection resulted in higher activities than conventional injection at all doses (Fig. 2): 2-fold higher ($P = 0.06$) at 1×10^{12} vp/kg, 2.5-fold higher ($P = 0.009$) at 5×10^{12} vp/kg, and 1.5-fold higher ($P = 0.06$) at 1×10^{13} vp/kg. To determine the amount of vector genomes taken up by the

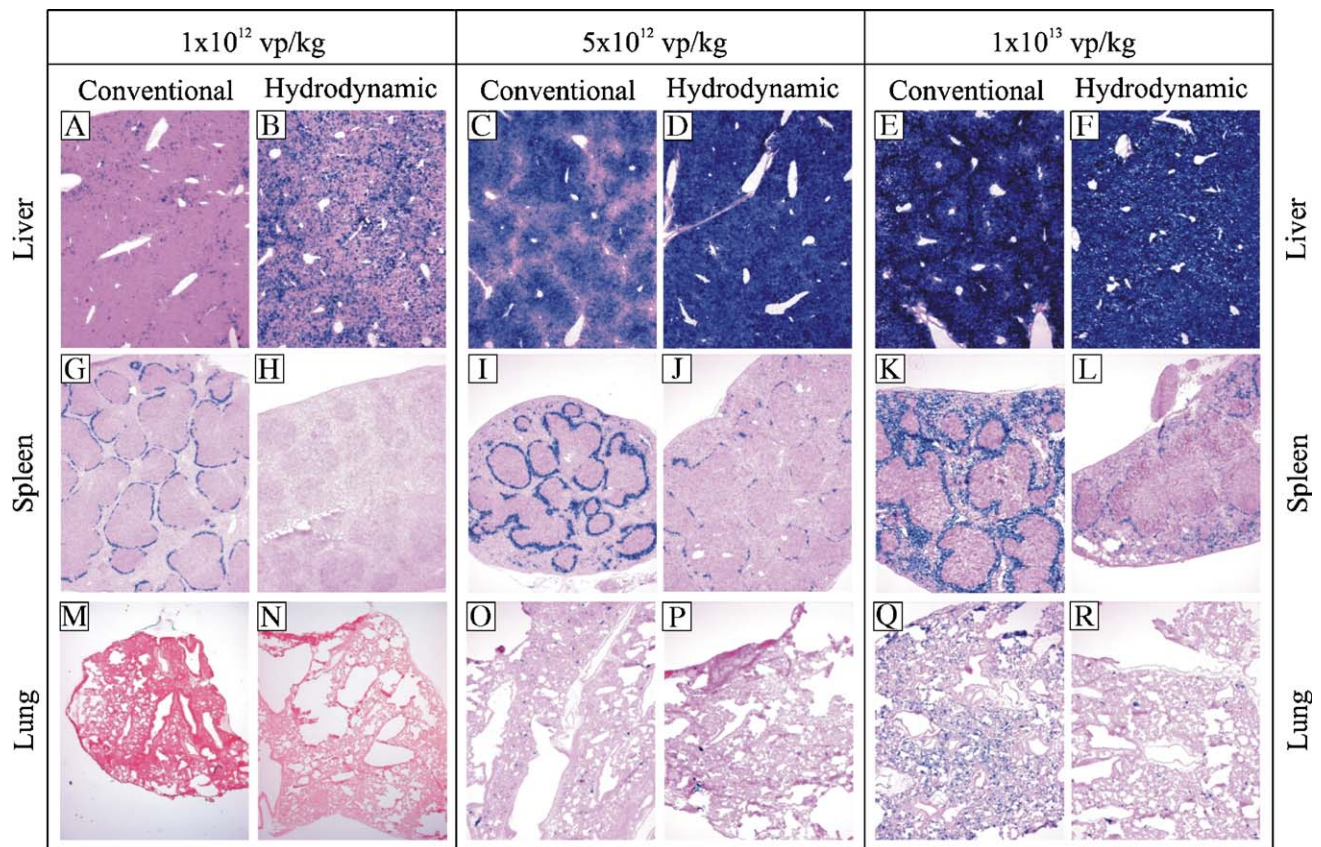


FIG. 1. X-gal histochemistry 48 h following conventional or hydrodynamic injection of HD $\Delta 28E4$ LacZ into mice. Liver following (A) conventional or (B) hydrodynamic injection of 1×10^{12} vp/kg. Liver following (C) conventional or (D) hydrodynamic injection of 5×10^{12} vp/kg. Liver following (E) conventional or (F) hydrodynamic injection of 1×10^{13} vp/kg. Spleen following (G) conventional or (H) hydrodynamic injection of 1×10^{12} vp/kg. Spleen following (I) conventional or (J) hydrodynamic injection of 5×10^{12} vp/kg. Spleen following (K) conventional or (L) hydrodynamic injection of 1×10^{13} vp/kg. Lung following (M) conventional or (N) hydrodynamic injection of 1×10^{12} vp/kg. Lung following (O) conventional or (P) hydrodynamic injection of 5×10^{12} vp/kg. Lung following (Q) conventional or (R) hydrodynamic injection of 1×10^{13} vp/kg. The results from one representative mouse from each treatment group are shown. The three organs shown for each injection condition are from the same mouse.

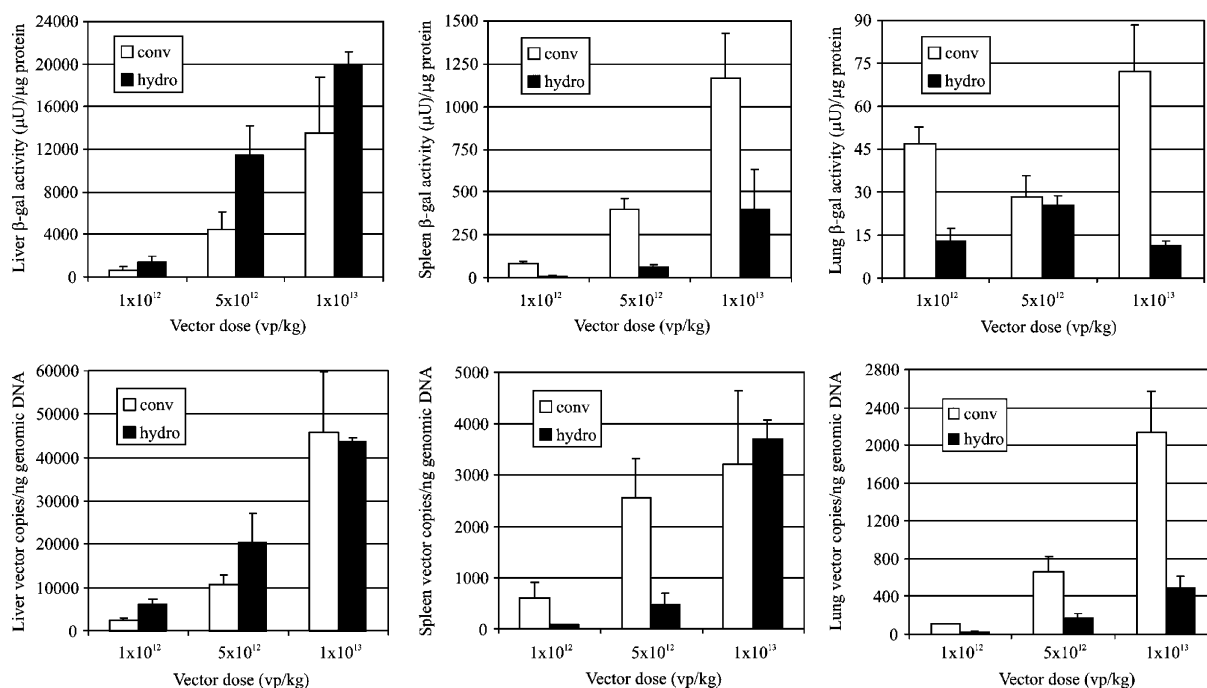


FIG. 2. β -Galactosidase activity and vector genome copy number in the liver, spleen, and lungs of mice 48 h following conventional (conv) or hydrodynamic (hydro) injection of HD Δ 28E4LacZ. Background β -galactosidase activity was ($n = 3$, no vector): liver, $12.5 \pm 2.5 \mu\text{U}/\mu\text{g}$ protein; spleen, $9.3 \pm 0.5 \mu\text{U}/\mu\text{g}$ protein; lung, $6.7 \pm 2.7 \mu\text{U}/\mu\text{g}$ protein. Vector genome was undetectable in mock-injected animals.

liver, we extracted total DNA and subjected it to quantitative real-time PCR. The number of vector genomes per nanogram of DNA following hydrodynamic injection was 2.7-fold higher ($P = 0.002$) at 1×10^{12} vp/kg and 1.9-fold higher ($P = 0.04$) at 5×10^{12} vp/kg compared to conventional injection (Fig. 2). At 1×10^{13} vp/kg, the number of vector genomes per nanogram of DNA was no different following hydrodynamic or conventional injection ($P = 0.4$) (Fig. 2), likely because this high dose was saturating.

Splenic Vector Uptake

In contrast to the liver, conventional injection at all doses (1×10^{12} , 5×10^{12} , and 1×10^{13} vp/kg) (Figs. 1G, 1I, and 1K) appeared to yield qualitatively more β -galactosidase-positive cells in the spleen than hydrodynamic injection (Figs. 1H, 1J, and 1L) as determined by X-gal histochemistry. This was consistent with the β -galactosidase activity assay, which revealed that conventional injection resulted in higher activities than hydrodynamic injection at all doses (Fig. 2), an increase of 8.2-fold ($P = 0.0001$) at 1×10^{12} vp/kg, 6.6-fold ($P = 0.0005$) at 5×10^{12} vp/kg, and 2.9-fold ($P = 0.01$) at 1×10^{13} vp/kg. Compared to hydrodynamic injection, the number of vector genomes per nanogram of DNA following conventional injection was also 2.7-fold higher ($P = 0.002$) at 1×10^{12} vp/kg and 9.4-fold higher ($P = 0.08$) at 5×10^{12} vp/kg as determined by quantitative real-time

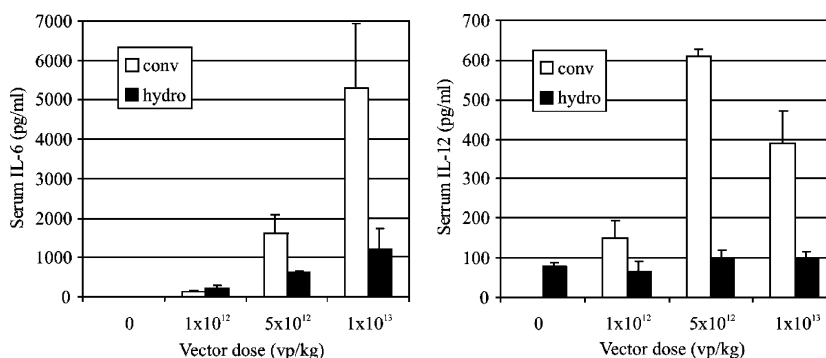
PCR (Fig. 2). The number of vector genomes per nanogram of DNA was no different following hydrodynamic or conventional injection of 1×10^{13} vp/kg ($P = 0.3$) (Fig. 2).

Pulmonary Vector Uptake

Qualitatively, very few β -galactosidase-positive cells were visible in the lungs at 1×10^{12} or 5×10^{12} vp/kg following either conventional (Figs. 1M and 1O) or hydrodynamic (Figs. 1N and 1P) injection as determined by X-gal histochemistry. However, at a dose of 1×10^{13} vp/kg, conventional injection (Fig. 1Q) yielded more β -galactosidase-positive cells than hydrodynamic injection (Fig. 1R). Quantitatively, as determined by β -galactosidase activity assay, conventional injection resulted in a 3.6-fold higher ($P = 0.0006$) and 6.4-fold higher ($P = 0.002$) level of activity than hydrodynamic injection at a dose of 1×10^{12} and 1×10^{13} vp/kg, respectively (Fig. 2). We observed no difference ($P = 0.3$) in activity in the lungs at a dose of 5×10^{12} vp/kg (Fig. 2). With respect to vector genomes per nanogram of DNA, conventional injection resulted in higher levels than hydrodynamic injection at all doses as determined by quantitative real-time PCR (Fig. 2): 3.6-fold higher ($P = 0.00006$) at 1×10^{12} vp/kg, 3.8-fold higher ($P = 0.003$) at 5×10^{12} vp/kg, and 4.3-fold higher ($P = 0.002$) at 1×10^{13} vp/kg.

Taken together, we interpret the results presented in Figs. 1 and 2 to suggest that hydrodynamic injection alters vector biodistribution, resulting in higher effi-

FIG. 3. Serum IL-6 and IL-12 in mice 6 h following conventional (conv) or hydrodynamic (hydro) injection of HDAd.



ciency hepatic transduction at the expense of extrahepatic transduction as evident by the lower efficiency of vector uptake and transduction in the spleen and lungs.

Proinflammatory Cytokines

Systemic intravascular administration of Ad-based vectors results in activation of the innate inflammatory response, marked by elevations of proinflammatory cytokines, the magnitude of which is dose-dependent [10,11,15]. Therefore, we compared the levels of the proinflammatory cytokines IL-6, IL-12, and TNF α in the mice presented above at 6 and 48 h postinjection. Serum IL-6 was undetectable following injection of vehicle alone under either conventional or hydrodynamic injection. At a dose of 1×10^{12} vp/kg, hydrodynamic injection resulted in a 1.6-fold higher ($P = 0.05$) level of IL-6 compared to conventional injection at 6 h (Fig. 3). At 5×10^{12} and 1×10^{13} vp/kg, conventional injection resulted in 2.6- ($P = 0.01$) and 4.4-fold higher ($P = 0.007$) levels of IL-6, respectively, than hydrodynamic injection at 6 h (Fig. 3). IL-6 returned to undetectable levels by 48 h for all doses and injection conditions.

For serum IL-12, hydrodynamic injection of vehicle alone resulted in an elevation from undetectable to 77.6 ± 10 pg/ml at 6 h (Fig. 3). In contrast, conventional injection of vehicle alone resulted in no detectable IL-12 at 6 h, indicating that hydrodynamic

injection alone increased IL-12 (Fig. 3). Interestingly, at all doses, conventional injection resulted in higher levels of IL-12 than hydrodynamic injection at 6 h (Fig. 3); 2.3-fold higher ($P = 0.02$) at 1×10^{12} vp/kg, 6.1-fold higher ($P = 0.000003$) at 5×10^{12} vp/kg, and 4-fold higher ($P = 0.002$) at 1×10^{13} vp/kg. IL-12 returned to undetectable levels by 48 h for all doses and injections.

We observed no elevation in serum TNF α at any dose under either injection condition (data not shown). This is consistent with published studies indicating little to no elevation in serum TNF α in mice, nonhuman primates, or humans following systemic administration of Ad in other studies [11,12,15,16].

Because hydrodynamic injection involves injection of about 2.5 to 3.0 ml into a mouse, possible dilution effects from the large volume may result in underestimations of the serum cytokines levels. To investigate this, we hydrodynamically injected four mice with vehicle only and measured the hematocrit as well as the serum levels of albumin and total protein 6 h postinjection and compared them to preinjection values obtained 21 days previously. All three parameters were reduced 6 h post-hydrodynamic injection compared to baseline values: albumin decreased 9.1% ($P = 0.02$), total protein decreased 8% ($P = 0.03$), and the hematocrit decreased 7.6% ($P = 0.03$) (Table 1). However, these reductions were small relative to the differences observed for IL-6 and IL-12. Therefore, a

TABLE 1: Dilution effect of hydrodynamic injection

Mouse	Albumin (g/dl)		Total protein (g/dl)		Hematocrit (%)	
	Pre ^a	Post ^b	Pre ^a	Post ^b	Pre ^a	Post ^b
1	2.8	2.4	5.1	4.4	48	44
2	2.7	2.7	5.0	4.9	46	41
3	2.8	2.6	5.0	4.8	48	45
4	2.7	2.3	4.8	4.2	43	41
Mean \pm SD	2.75 ± 0.1	2.5 ± 0.2	5.0 ± 0.1	4.6 ± 0.3	46.3 ± 2.4	42.8 ± 2.1

^a Baseline value obtained 21 days pre-hydrodynamic injection.

^b Value obtained 6 h post-hydrodynamic injection.

dilution effect of hydrodynamic injection does not account for the lower levels of IL-6 and IL-12 observed compared to conventional injection. That the excess volume introduced by hydrodynamic injection is rapidly excreted is consistent with a previous study [17].

Taken together, we interpret these results to suggest that systemic HDAd administered by hydrodynamic injection results in a less severe elevation in serum IL-6 and IL-12 compared to administration by conventional injection.

Kupffer Cells

Kupffer cells (KC) of the liver are a barrier to efficient hepatocyte transduction by avidly sequestering Ad following systemic intravascular administration, at least in mice [5,6], and this has been implicated to play a role in innate inflammatory response activation [6,18]. Therefore, we next investigated the effect of hydrodynamic injection on vector uptake by KC by injecting fluorescently labeled HD Δ 28E4LacZ either hydrodynamically or conventionally into mice at a dose of 1×10^{12} vp/kg ($n = 3$ per injection condition). We sacrificed the mice 1 h postinjection and harvested the livers for fluorescence microscopy to localize the vector and the KC. The results revealed comparable and extensive colocalization of KC (green) and vector (red) following either conventional or hydrodynamic injection. The results of two representative mice are presented in Fig. 4. Specifically, the percentage of KC that appeared not colocalized with vector was comparable

following either conventional (5/45 or 11.1%) or hydrodynamic (6/65 or 11.5%) injection. These results indicate that hydrodynamic injection does not alter the number of KC that take up vector compared to conventional injection.

Duration of Transgene Expression

HDAd have been shown to mediate long-term transgene expression following hepatocyte transduction [1,2]. Therefore, we next investigated whether hydrodynamic injection would affect the duration of expression by injecting three mice with 1×10^{12} vp/kg of HD Δ 21.7E4PEPCK-hFIX-WL, a HDAd bearing a liver-restricted expression cassette for human coagulation factor IX (hFIX), under either hydrodynamic or conventional conditions. As shown in Fig. 5, both injection conditions resulted in stable, high-level transgene expression for at least 190 days, indicating that hydrodynamic injection did not compromise the duration of expression from the HDAd. Consistent with the results presented in Fig. 2, hydrodynamic injection resulted in 2.8-fold higher plasma hFIX compared to conventional injection.

DISCUSSION

It has been proposed that the large volume rapidly delivered by hydrodynamic injection results in accumulation of excess fluid in the liver, as it is the largest organ in the body with an expandable architecture. Consequently, the liver fenestrations are enlarged due to

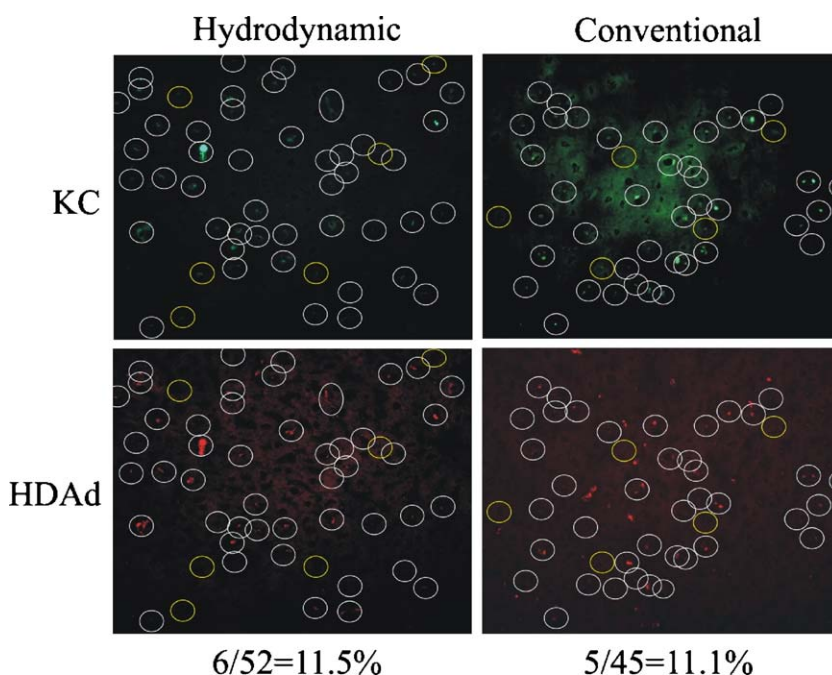


FIG. 4. Fluorescence microscopy of the liver from representative mice 1 h postinjection with 1×10^{12} vp/kg of Cy3-labeled HD Δ 28E4LacZ (HDAd) under conventional or hydrodynamic conditions. Locations of KC (green) are indicated by circles. KC that do not appear colocalized with HDAd (red) are indicated by yellow circles.

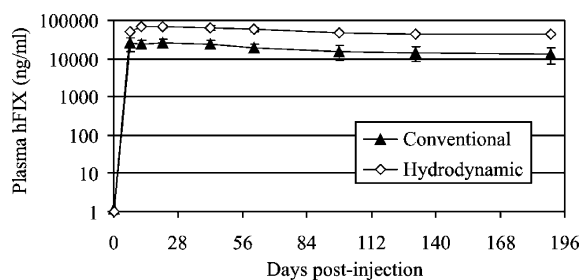


FIG. 5. The mean \pm SD plasma levels of hFIX from mice ($n = 3$) following conventional or hydrodynamic injection of 1×10^{12} vp/kg of HD Δ 21. 7E4PEPCK-hFIX.

the high intrahepatic pressure [13]. Since the liver fenestrations are ≤ 100 nm in diameter, their enlargement by hydrodynamic injection may result in increased extravasation of the 100 nm HDAd virion into the liver parenchyma, thus increasing the likelihood of hepatocyte transduction. Indeed, we demonstrate that systemic hydrodynamic injection of HDAd results in higher efficiency of hepatic vector uptake compared to conventional injection. Consistent with our results is a study showing a direct positive relationship between the size of the fenestrations and the hepatocyte transduction efficiency by systemic Ad in rabbits [20]. Furthermore, enlargement of the fenestrations with sodium decanoate resulted in increased hepatocyte transduction by Ad [20].

The spleen and the lung are major sites of vector deposition following systemic administration of Ad [11,12,15]. Our results also show that hydrodynamic injection was accompanied by reductions in splenic and pulmonary vector uptake. These results were perhaps not surprising because it is reasonable to assume that if more of the input dose were directed to the liver, then less would be available for extrahepatic uptake.

An important finding is that elevations in the proinflammatory cytokines IL-6 and IL-12, sensitive markers of the inflammatory response following systemic administration of Ad [10–12,16,21], were less severe following hydrodynamic than following conventional injection. Significantly, although hydrodynamic injection itself results in a modest elevation in serum IL-12, it appears to negate vector-mediated elevation in IL-12 because, unlike conventional injection, serum IL-12 levels were no longer correlative to vector dose or level of hepatic transduction. Taken together, these results suggest that high efficiency hepatic transduction does not, at least alone, necessarily provoke a potent inflammatory response. Instead, the results suggest that extrahepatic vector uptake may play a major role in the severity of the inflammatory response. Indeed, numerous studies have implicated extrahepatic vector uptake to play an important role in the acute inflammatory response to systemic Ad. For example, vector uptake and activation to secrete

proinflammatory cytokines by macrophages and dendritic cells in the spleen and the lungs have been implicated [11,12,18]. Vector transduction of endothelial cells [9,22] and peripheral mononuclear cells [23] and Ad-mediated complement activation [24,25] have all been implicated to play a role in acute toxicity. Several studies also suggest that antibodies, both neutralizing and naturally occurring (nonspecific cross-reacting), may contribute to acute toxicity [5,6,16,24]. For example, systemic vector administration into nonhuman primates resulted in significantly higher IL-6 levels in animals with neutralizing anti-Ad antibodies compared to naïve animals [16]. An important implication of our study is that factors or conditions that result in a decrease in the size of the hepatic sinusoidal fenestrations, such as cirrhosis, hepatic metastasis, and aging [26], may decrease the therapeutic index of Ad by decreasing hepatic, while increasing extrahepatic vector uptake. These factors may need to be taken into consideration in assessing the risk:benefit ratio.

KC of the liver have been implicated in acting as a barrier to efficient hepatocyte transduction because prior depletion of KC with chlodronate liposomes results in higher efficiency hepatic transduction [5,6]. However, the depletion of extrahepatic reticuloendothelial cells with systemic chlodronate liposomes cannot be ruled out and these and other cell types may also contribute to the barrier to hepatocyte transduction. Although our results suggest that avid uptake of HDAd by KC is unaffected by hydrodynamic injection, they also suggest that extrahepatic vector uptake also contributes significantly to the barrier to efficient hepatocyte transduction. Indeed, although the liver takes up more vector than any other individual organ following systemic injection, the total amount of vector that is distributed throughout the body in mice [11], in nonhuman primates [12,16], and in the one human studied [15] is significant. It also has been suggested that vector uptake by KC plays a major role in the activation of the innate inflammatory response [6,18]. However, our results suggest that vector uptake by KC, at least alone, is not sufficient to provoke a severe proinflammatory cytokine response because the levels of IL-6 and IL-12 do not appear to correlate with vector uptake by KC. Indeed the consequence of vector uptake by KC on the severity of the acute innate inflammatory response is controversial and remains to be fully elucidated because most studies in mice utilizing KC depletion prior to Ad administration did not investigate cytokine response [27–29]. Of the studies that did, systemic injection of first-generation Ad into mice depleted of KC nearly eliminated production of TNF α but resulted in a more robust IL-6 increase and did not affect NF- κ B compared to mice with KC [30]. In another study, depletion of KC in mice prior to systemic injection of HDAd appeared to have no effect on the serum levels of

IL-2, IL-4, IL-5, IL-6, TNF- α , and IFN- γ [31]. It is also important to note that systemic injection of low vector doses into nonhuman primates and mice that result in low efficiency hepatocyte transduction likely yield substantial vector uptake by KC because they preferentially take up vector before hepatocytes [5,6]. Yet, these animals exhibit little, if any, manifestations of acute toxicity. Taken together, these studies also suggest that vector uptake by KC, at least alone, may not necessarily provoke a potent inflammatory response. Indeed it has been suggested that KC may, in fact, play a protective role [32]. However, it should be noted that the method of colocalizing KC and Ad that we, and others, have used is not quantitative because the amount of HDAd taken up per KC cannot be ascertained. Therefore, it is possible that less HDAd is taken up per KC following hydrodynamic than following conventional injection and that this might account, at least in part, for the higher efficiency of hepatic transduction and reduced proinflammatory cytokine levels.

Ultimately, the precise mechanism responsible for activation of the acute inflammatory response by systemic Ad is multifactorial and complex and remains to be fully elucidated. However, regardless of the precise mechanism, it is likely that a threshold of innate immune activation must first be attained, as a consequence of high dose and systemic exposure of the vector to many cell types and blood-borne components, before severe and lethal acute toxicity manifests. Nevertheless, one important implication of this study is that high efficiency hepatic transduction by HDAd does not, at least alone, necessarily result in potent activation of the innate inflammatory response, but that systemic vector dissemination may play a key role in this regard. Therefore, one approach to increase the therapeutic index of HDAd may be to alter the vector biodistribution to favor hepatic transduction while minimizing systemic dissemination. While systemic hydrodynamic injection as performed in the mouse is not feasible in larger animals, methods of localized hydrodynamic injection directly into the liver may offer this potential [33,34]. Alternatively, enlargement of the hepatic sinusoidal fenestrations by pharmacological means may also be an option [20]. Perhaps the ultimate approach may be to alter the tropism of the vector to target exclusively hepatocytes. Successful development of these or other strategies should greatly increase the therapeutic index of these vectors.

MATERIALS AND METHODS

Vectors. HD Δ 28E4LacZ contains a MCMV-LacZ expression cassette [14]. HD Δ 21.7E4PEPCK-hFIX-WL contains a hFIX expression cassette composed of the following elements (from 5' to 3'): a liver-restricted rat phosphoenolpyruvate carboxykinase promoter [35], the ApoAI intron, the hFIX cDNA, the woodchuck hepatitis virus posttranscriptional regulatory element, the ApoE locus control region, and the human

growth hormone poly(A). Both HDAd's were produced in 116 cells [14] with the helper virus AdNG163 [36] as described in detail elsewhere [14,37]. Characterization of both vectors, as described elsewhere [14], revealed the expected genomic structure and a level of helper virus contamination of <0.05%.

Mice and injections. Nine- to 12-week-old male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME, USA) were used for all the experiments. HDAd was diluted in sterile Ringer's solution (147 mM NaCl, 4 mM KCl, 1.13 mM CaCl₂) prewarmed at 37°C and injected into the tail vein. Conventional injections were performed with a volume of 200 μ l. Hydrodynamic injections were performed with a volume of 100 ml/kg using a 27-gauge needle at a rate of 0.4 ml/s. Unless otherwise stated all treatment groups consisted of at least three mice. For mice injected with HD Δ 28E4LacZ, blood was collected retro-orbitally for analyses at 6 h postinjection and at the time of sacrifice at 48 h postinjection. Upon sacrifice, the liver, spleen, and lung were harvested and kept on dry ice or at -80°C until analyses. Mice injected with HD Δ 21.7E4PEPCK-hFIX-WL were bled from the tail vein at various times postinjection and blood samples were collected into 1/10 volume of 3.2% sodium citrate buffer to prevent coagulation and plasma was prepared by centrifugation. Plasma was frozen immediately and stored at -80°C until analyses.

Analyses of tissue and blood. X-gal histochemistry was performed on liver, spleen, and lung from mice as described previously [10]. Total protein was extracted from the liver, lung, and spleen and the β -galactosidase activity was determined using the β -Galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega, Madison, WI, USA) following quantification using the Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Total DNA was extracted from liver, spleen, and lung using the QIAamp DNA extraction kit (Qiagen) and quantitated by absorbance at 260 nm. Quantitative real-time PCR was performed using the LightCycler FastStart DNA Master SYBR Green I (Roche) in a total volume of 20 μ l with 2 μ l of template DNA, 4 mM MgCl₂, and 5 μ M each HDAd-specific primer (5'-TCTGAA-TAATTTGTGTACTCATAGCGCG-3' and 5'-CCCATAAGCTCCTTT-TAACTTGTTAAAGTC-3'). Cycling conditions consisted of 95°C for 10 min followed by 45 cycles at 95°C for 10 s, 60°C for 7 s, and 72°C for 20 s. Serial dilutions of a plasmid bearing the PCR target sequence were used as a control to determine the amounts of HDAd and results were analyzed with LightCycler software version 3.5 (Roche). Serum levels of IL-6, IL-12, and TNF α were determined by ELISA (Biosource) according to the manufacturer's protocol. Plasma hFIX antigen levels were measured by ELISA (Enzyme Research Laboratories, South Bend, IN, USA) according to the manufacturer's protocol. *In situ* localization by fluorescence microscopy of KC and HDAd was performed as described elsewhere [19]. Statistical analyses were performed with the *t* test.

ACKNOWLEDGMENTS

This work was support by the National Institutes of Health (P50 HL59314) and the Texas Affiliate of the American Heart Association (0465102Y). We thank the Morphology Core Laboratory of the Gulf Coast Digestive Disease Center and Dorene M. Rudman for the enzyme histochemistry and Terry Bertin for assistance with the real-time PCR assays.

RECEIVED FOR PUBLICATION FEBRUARY 23, 2005; ACCEPTED MARCH 3, 2005.

REFERENCES

- Ng, P., and Graham, F. L. (2004). Helper-dependent adenoviral vectors for gene therapy. In *Gene Therapy: Therapeutic Mechanisms and Strategies*, 2nd ed. (N. S. Templeton, Ed.), pp. 53–70. Dekker, New York.
- Palmer, D., and Ng, P. (2005). Helper-dependent adenoviral vectors for gene therapy. *Hum. Gene Ther.* **16**: 1–16.
- Morrall, N., et al. (1998). High doses of a helper-dependent adenoviral vector yield supraphysiological levels of α 1-antitrypsin with negligible toxicity. *Hum. Gene Ther.* **9**: 2709–2716.

4. Muruve, D. A., Barnes, M. J., Stillman, I. E., and Libermann, T. A. (1999). Adenoviral gene therapy leads to rapid induction of multiple cytokines and acute neutrophil-dependent hepatic injury in vivo. *Hum. Gene Ther.* **10**: 965–976.
5. Tao, N., et al. (2001). Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. *Mol. Ther.* **3**: 28–35.
6. Schiedner, G., Hertel, S., Johnston, M., Dries, V., van Rooijen, N., and Kochanek, S. (2003). Selective depletion or blockade of Kupffer cells leads to enhanced and prolonged hepatic transgene expression using high-capacity adenoviral vectors. *Mol. Ther.* **7**: 35–43.
7. Sullivan, D. E., et al. (1997). Liver-directed gene transfer in non-human primates. *Hum. Gene Ther.* **8**: 1195–1206.
8. Nunes, F. A., Furth, E. E., Wilson, J. M., and Raper, S. E. (1999). Gene transfer into the liver of nonhuman primates with E1-deleted recombinant adenoviral vectors: safety of readministration. *Hum. Gene Ther.* **10**: 2515–2526.
9. Morral, N., et al. (2002). Lethal toxicity, severe endothelial injury, and a threshold effect with high doses of an adenoviral vector in baboons. *Hum. Gene Ther.* **13**: 143–154.
10. Brunetti-Pierri, N., Palmer, D. J., Beaudet, A. L., Carey, D., Finegold, M., and Ng, P. (2004). Acute toxicity following high-dose systemic injection of helper-dependent adenoviral vectors into non human primates. *Hum. Gene Ther.* **15**: 35–46.
11. Zhang, Y., et al. (2001). Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. *Mol. Ther.* **3**: 697–707.
12. Schnell, M. A., et al. (2001). Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. *Mol. Ther.* **3**: 708–722.
13. Zhang, G., et al. (2004). Hydroporation as the mechanism of hydrodynamic delivery. *Gene Ther.* **8**: 675–682.
14. Palmer, D. J., and Ng, P. (2003). Improved system for helper-dependent adenovirus vector production. *Mol. Ther.* **8**: 846–852.
15. Raper, S. E., et al. (2003). Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol. Genet. Metab.* **80**: 148–158.
16. Varnaski, A. N., et al. (2002). Preexisting immunity to adenovirus in rhesus monkeys fails to prevent vector-induced toxicity. *J. Virol.* **76**: 5711–5719.
17. Rossmannith, W., Chabicovsky, M., Herkner, K., and Schulte-Hermann, R. (2002). Cellular gene dose and kinetics of gene expression in mouse livers transfected by high-volume tail-vein injection of naked DNA. *DNA Cell Biol.* **11**: 847–853.
18. Smith, J. S., Tian, J., Muller, J., and Byrnes, A. P. (2004). Unexpected pulmonary uptake of adenovirus vectors in animals with chronic liver disease. *Gene Ther.* **11**: 431–438.
19. Mok, H., Palmer, D. J., Ng, P., and Barry, M. A. (2005). Evaluation of polyethylene glycol (PEG)-modification of first-generation and helper-dependent adenoviral vectors to reduce innate immune responses. *Mol. Ther.* **1**: 66–79.
20. Lievens, J., et al. (2004). The size of sinusoidal fenestrae is a critical determinant of hepatocyte transduction after adenoviral gene transfer. *Gene Ther.* **11**: 1523–1531.
21. Ben-Gary, H., McKinney, R. L., Rosengart, T., Lesser, M. L., and Crystal, R. G. (2002). Systemic interleukin-6 responses following administration of adenovirus gene transfer vectors to humans by different routes. *Mol. Ther.* **6**: 287–297.
22. Ramalingam, R., Rafii, S., Worgall, S., Hackett, N. R., and Crystal, R. G. (1999). Induction of endogenous genes following infection of human endothelial cells with an E1⁻E4⁺ adenovirus gene transfer vector. *J. Virol.* **73**: 10183–10190.
23. Higginbotham, J. N., Seth, P., Blaes, R. M., and Ramsey, E. J. (2002). The release of inflammatory cytokines from human peripheral blood mononuclear cells in vitro following exposure to adenovirus variants and capsid. *Hum. Gene Ther.* **13**: 129–141.
24. Cichon, G., et al. (2001). Complement activation by recombinant adenoviruses. *Gene Ther.* **8**: 1794–1800.
25. Jiang, H., Wang, Z., Serra, D., Frank, M. M., and Amalfitano, A. (2004). Recombinant adenovirus vectors activate the alternative complement pathway, leading to the binding of human complement protein C3 independent of anti-Ad antibodies. *Mol. Ther.* **6**: 1140–1142.
26. Braet, F., and Wisse, E. (2002). Structural and functional aspects of liver sinusoidal endothelial cell fenestrae: a review. *Comp. Hepatol.* **1**: 1–17.
27. Kuzmin, A. I., Finegold, M. J., and Eisensmith, R. C. (1997). Macrophage depletion increases the safety, efficacy and persistence of adenovirus-mediated gene transfer in vivo. *Gene Ther.* **4**: 309–316.
28. Wolff, G., Worgall, S., van Rooijen, N., Song, W. R., Harvey, B. G., and Crystal, R. G. (1997). Enhancement of in vivo adenovirus-mediated gene transfer and expression by prior depletion of tissue macrophages in the target organ. *J. Virol.* **71**: 624–629.
29. Kuzmin, A. I., Galenko, O., and Eisensmith, R. C. (2001). An immunomodulatory procedure that stabilizes transgene expression and permits readministration of E1-deleted adenovirus vectors. *Mol. Ther.* **3**: 293–301.
30. Liber, A., et al. (1997). The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. *J. Virol.* **71**: 8798–8807.
31. Schiedner, G., et al. (2003). A hemodynamic response to intravenous adenovirus vector particles is caused by systemic Kupffer cell-mediated activation of endothelial cells. *Hum. Gene Ther.* **14**: 1631–1641.
32. Jooss, K., and Chirmule, N. (2003). Immunity to adenovirus and adeno-associated viral vectors: implications for gene therapy. *Gene Ther.* **10**: 955–963.
33. Zhang, G., Vargo, D., Budker, B., Armstrong, N., Knechtle, S., and Wolff, J. A. (1997). Expression of naked plasmid DNA injected into the afferent and efferent vessels of rodent and dog livers. *Hum. Gene Ther.* **8**: 1763–1772.
34. Eastman, S. J., et al. (2002). Development of catheter-based procedure for transducing the isolated rabbit liver with plasmid DNA. *Hum. Gene Ther.* **13**: 2065–2077.
35. Beale, E. G., Clouthier, D. E., and Hammer, R. E. (1992). Cell-specific expression of cytosolic phosphoenolpyruvate carboxykinase in transgenic mice. *FASEB J.* **6**: 3330–3337.
36. Palmer, D. J., and Ng, P. (2004). Physical and infectious titers of helper-dependent adenoviral vectors: a method of direct comparison to the Adenovirus Reference Material. *Mol. Ther.* **10**: 792–798.
37. Ng, P., Parks, R. J., and Graham, F. L. (2002). Methods for the preparation of helper-dependent adenoviral vectors. *Methods Mol. Med.* **69**: 371–388.