Short-term correction of factor VIII deficiency in a murine model of hemophilia A after delivery of adenovirus murine factor VIII *in utero*

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Edited by Irving L. Weissman, Stanford University School of Medicine, Stanford, CA, and approved September 23, 1999 (received for review August 17, 1999)

Development of *in utero* gene transfer approaches may provide therapies for genetic disorders with perinatal morbidity. In hemophilia A, prenatal and postnatal bleeding may be catastrophic, and modest increments in factor VIII (FVIII) activity are therapeutic. We performed transuterine i.p. gene transfer at day 15 of gestation in a murine model of hemophilia A. Normal, carrier (X_HX), and FVIII-deficient (X_HY and X_HX_H) fetuses injected with adenoviral vectors carrying luciferase or β-galactosidase reporter genes showed high-level gene expression with 91% fetal survival. The live-born rates of normal and FVIII-deficient animals injected *in utero* with adenovirus murine FVIII (3.3×10^5 plaque-forming units) was 87%. FVIII activity in plasma was 50.7 ± 10.5% of normal levels at day 2 of life, 7.2 ± 2.2% by day 15 of life, and no longer detectable at day 21 of life in hemophilic animals. Injection of higher doses of murine FVIII adenovirus at embryonic day 15 produced supranormal levels of FVIII activity in the neonatal period. PCR analysis identified viral genomes primarily in the liver, intestine, and spleen, although adenoviral DNA was detected in distal tissues when higher doses of adenovirus were administered. These studies show that transuterine i.p. injection of adenoviral vectors produces therapeutic levels of circulating FVIII throughout the neonatal period. The future development of efficient and persisting vectors that produce long-term gene expression may allow for *in utero* correction of genetic diseases originating in the fetal liver, hematopoietic stem cells, as well as other tissues.

gene therapy

Hemophilia A, a deficiency of factor VIII (FVIII), is an X-linked disorder affecting 1 in 5,000 males (1). Individuals with severe hemophilia (<1% FVIII activity) have spontaneous hemorrhages in joints, in muscle, in internal organs, and intracranially. Infusions of pooled human FVIII are effective but are associated with transmission of viral disease. Administration of recombinant FVIII reduces this risk but costs considerably more (2).

Many features of hemophilia A make this disorder an attractive target for gene transfer-based therapies. Expression of as little as 1–5% of normal FVIII levels would reduce spontaneous bleeding in severe hemophilia patients. Short-term expression of FVIII would be clinically useful in patients with milder forms of disease and would benefit patients who need prophylactic FVIII infusion therapy for surgical and other interventions. Production of FVIII is not tissue restricted, and functional protein can be produced and secreted from a variety of cell types (3–5). A third advantage of hemophilia as a model for gene therapy is that precise regulation of the level of gene expression is not required, because supranormal levels are not associated with an increased risk of thrombosis. Finally, FVIII vectors may now be evaluated in recently developed murine models of hemophilia A (6, 7).

The transduction of self-renewing progenitor cells will be required for the durable correction of FVIII deficiency. The delivery and expression of FVIII *in utero* may allow transduction of stem cell populations that are present in greater numbers during fetal life. Several groups have reported successful gene transfer into fetal tissues *in vivo* (8–14). Both retroviral and adenoviral vectors have been introduced into the airways of ovine fetuses with subsequent gene expression in airway epithelial cells (8–12). Replication-deficient recombinant adenoviruses show hepatic tropism and have been studied extensively in mammalian systems (15). Advantages of adenoviral vectors are that they accommodate relatively large gene inserts, can be grown to high titers, and efficiently infect both dividing and nondividing cells of many organs (15). However, phenotypic correction of a genetic disorder by *in utero* i.p. delivery of adenoviral vectors has not been described. The tissue distribution, level, and duration of gene expression after adenoviral-mediated prenatal gene delivery are yet to be defined.

We have used a murine model of hemophilia A produced by targeted disruption of exon 17 as a model for *in utero* adenoviral-mediated FVIII gene delivery (6). The FVIII activity in carrier females (X_HX) is on average 74% of normal levels, whereas affected (hemophilic) animals (X_HY, X_HX_H) have <1% FVIII activity (6). Male mice carrying the exon 17 disruption (X_HY) and females homozygous for the exon 17 disruption (X_HX_H) develop exsanguinating bleeding after minimal trauma such as tail clipping unless cautery is performed (6, 7). In these studies, we have (*i*) determined the sites of viral transduction after *in utero* i.p. delivery of adenoviral vectors carrying reporter genes and (*ii*) determined that therapeutic FVIII levels in neonates are achieved after adenoviral-mediated *in utero* delivery of the murine FVIII (mFVIII) cDNA without significant toxicity.

Materials and Methods

Animal Procedures. All animal procedures were approved by the University of California San Francisco Committee on Animal Research. Mice carrying targeted deletions of exon 17 of the FVIII gene have been described (6). Mice with homozygous deletions in the FVIII gene were bred with CD-1 mice (Charles River Breeding Laboratories). Carrier females (X_HX) were bred with affected males (X_HY). On day 15 of gestation, the females were anesthetized, and transuterine injection was performed as described (16). Control animals were either not injected or injected with 5 μl of normal saline (NS). Adenovirus stocks were diluted in NS, and 5–20 μl was delivered to the i.p. space.

Tissues from fetal and adult animals were obtained as described (16). Blood was obtained from adult animals by collection from the retroorbital plexus and transferred to tubes

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: FVIII, factor VIII; mFVIII, murine FVIII; Ad-, adenovirus-; CMV, cytomegalovirus; E*n*, early gene; RLU, relative light unit; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; NS, normal saline; pfu, plaque-forming unit.

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containing 0.1 M sodium citrate in a 9:1 ratio. Neonatal blood was collected from the facial vein of pups.

Construction and Purification of Recombinant Adenoviral Vectors. Total RNA from murine liver was isolated, and the poly(A) fraction was purified by using an mRNA isolation kit (CLONTECH). poly(A) RNA was primed with an oligo(dT) primer (0.5 μg), and first-strand synthesis was catalyzed by 200 units of Superscript II RNase H reverse transcriptase (GIBCO/BRL) at 42°C. The cDNA was then amplified with two specific oligonucleotides and Pfu DNA polymerase (Stratagene). A *Kpn*I site was introduced in frame with the Kozak sequence at 402 bp in the 5′ primer to facilitate cloning into the pAdCMVlink-1 plasmid vector. The 3′ primer included the poly(A) region at 7,471 bp. The amplified fragment was subcloned into pCR Script (Stratagene), and the cDNA sequence was verified. The B domain of mFVIII was deleted from the *Spe*I site at position 2,606 bp to the *Eco*RI site at position 4,675 bp; a 21-bp linker with a *Bam*HI site 5′ and *Eco*RI site 3′ was inserted to bring the remaining sequence in frame. This 4.9-kilobase B domain-deleted mFVIII cDNA was subcloned as a *Kpn*I–*Xho*I fragment into pAdCMVlink1. The resulting construct, pAdCMVmFVIII, was linearized by digestion with *Nhe*I and subsequently cotransfected with *Cla*I-digested adenovirus (Ad)5.dl7001 viral DNA into 293 cells by using calcium phosphate precipitation. Recombinant plaques were identified by restriction analysis of DNA and were subjected to three rounds of plaque purification. AdCa18Luc (ref. 17; a gift of Frank Graham, McMaster University, Hamilton, Ontario) and Ad-*LacZ* (a gift of J.-Y. Dong, Medical University of South Carolina, Charleston) are both early gene 1 (E1)- and E3-deleted serotype 5 replication-deficient recombinant adenoviral vectors containing the firefly luciferase gene and the β-galactosidase gene, respectively. Both reporter genes are driven by cytomegalovirus (CMV) promoter sequences. High-titer adenovirus was prepared and titered as described (16, 18). Virus stocks were screened for replication competent virions by examining for plaques on HeLa cells. Particle-to-plaque ratios were less than 100.

Preparation of Genomic DNA and PCR Analysis. Genomic DNA was prepared from fetal and newborn tail tips as described (19). Each sample (1 μl) was used in each PCR performed by standard methods. All primers for PCR analyses were synthesized in the University of California San Francisco DNA sequencing facility by standard techniques. The reactions were denatured for 5 min before amplification. A three-primer PCR was performed to identify exon-17-disrupted affected, carrier, and wild-type animals as described (7). To determine the sex of fetuses, PCR of Y-chromosome-specific sequences was used. Primers TD/BA-B1 (5′-GGCCGAATTCGTCAAGCGCCCCATGAATGCATT-3′) and TD/EA-R1 (5′-GGCCGAATTCTAGTTTGGGTATTTCTCTCTGTG-3′), modified from the previously published sequence encompassing the Sry HMG box region of the Y-chromosome (20), were synthesized by standard methods. A 30-cycle PCR amplification (94°C for 30 s, 55°C for 30 s, 72°C for 2 min) was performed with a 10-min 72°C extension on completion of the cycles. The presence of a 374-bp sequence after amplification indicated the presence of a Y chromosome. By combining these PCRs, fetal, newborn, and adult mouse genotypes (XX, XY, X_HX, X_HY, X_HX_H) could be definitively assigned.

Quantitation of Adenoviral Genomes in Tissues. Viral DNA was prepared from 200 μl of high-titer Ad-mFVIII by incubating at 37°C for 18 h with SDS and proteinase K. Lysates were extracted with phenol-chloroform/chloroform-isoamyl alcohol, and the DNA was precipitated. Genomic DNA was purified by standard techniques (19). To determine both the limits of viral genome detection by PCR and viral copy number, normal mouse genomic DNA was prepared, and viral DNA was added in a series of dilutions from 20 ng to 0.02 fg per 100 ng of genomic DNA. These samples were used to establish reference standards. A 5′ primer in the CMV promoter (5′-CCAAAATGTCGTAACAACTCCG-3′) and a 3′ primer in the mFVIII cDNA (5′-GGAAGAAATCTTGAGTCTGTATC-3′) were used to amplify adenoviral DNA with PCR cycles of 95°C for 45 s, 59°C for 40 s, and 72°C for 60 s (PCR product size = 597 bp). Genomic DNA samples from injected mice (100 ng) underwent 30 cycles of PCR amplification with 0.5 μCi of [γ-^{32}P]dCTP (Amersham Pharmacia) per reaction. PCR samples were resolved in either 4% polyacrylamide in TBE buffer (0.9 M Tris-borate/2 mM EDTA, pH 8.0) followed by autoradiography or by resolution in 3% (vol/vol) NuSieve 3:1 agarose (FMC) and then photographed. Mouse β-actin primers (forward primer: 5′-ATCTGGCACCACACCTTCTACA-ATGAGCTGCG-3′; reverse primer: 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′; 872-bp fragment) were used as an internal reference for PCR. Negative controls included DNA from uninjected carrier females and DNA from normal mice injected i.v. with an unrelated CMV-driven adenoviral vector (AdCa18Luc). The viral genome copy number per cell was estimated by PhosphorImager-based quantitation and comparison with the reference standards described above (Fuji Film BAS System).

Quantitation of Luciferase and Total Protein. Tissues were harvested and processed as described (16). Luciferase levels were detected by luminometry (Analytical Luminescence Laboratory, Sparks, MD) and reported as relative light units (RLU). Tissue luciferase levels were normalized by determining protein concentration of tissue extracts as described (16). All standards and samples were performed in duplicate, and means were calculated. Luciferase units are expressed as RLU per microgram tissue protein.

Histochemical Analysis. After cervical dislocation of the mother, whole fetuses were removed from the uterus, euthanized, and rinsed in PBS (4°C). Selected fetuses were placed in OCT compound (Sakura Finetek, Torrance, CA) and rapidly frozen in an isopentane/dry-ice bath. Tissue sections (10 μm) were cut and fixed. Whole fetuses were fixed [in 2% (vol/vol) neutral buffered formalin/0.2% glutaraldehyde/0.02% Nonidet P-40 in PBS (pH 7.4)] on ice for 2 h; tissue sections were fixed for 5 min. After rinsing in PBS (pH 7.4), tissues were placed in 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) staining solution (18). Tissue sections were stained at 37°C for 2 h; whole fetuses were stained for 18 h at 22°C. Sections were counterstained with nuclear fast red (Sigma).

Determination of FVIII Activity. FVIII levels were determined by COATEST (Diapharma, Molndal, Sweden), a chromogenic assay in which FVIII activity is measured based on the rate of activation of factor X by FVIIIa (as stated in the manufacturer's instructions). Normal mouse FVIII standards were generated after pooling plasma from 16 normal adult CD-1 mice. FVIII-deficient control plasma was made by pooling samples from 16 FVIII-deficient mice; plasma samples were stored at −80°C. Samples were read at 405 nm and analyzed (SOFTMAXPRO, Molecular Dynamics). All standards and samples were performed in duplicate, and means were calculated.

Statistical analysis mean and SEM were calculated by standard methods (21).

Results

Adenoviral-Mediated *LacZ* Expression Occurs Primarily in the Liver, Intestine, and Peritoneum of Injected Fetuses. Our initial goal was to assess the accuracy and reproducibility of fetal injection and

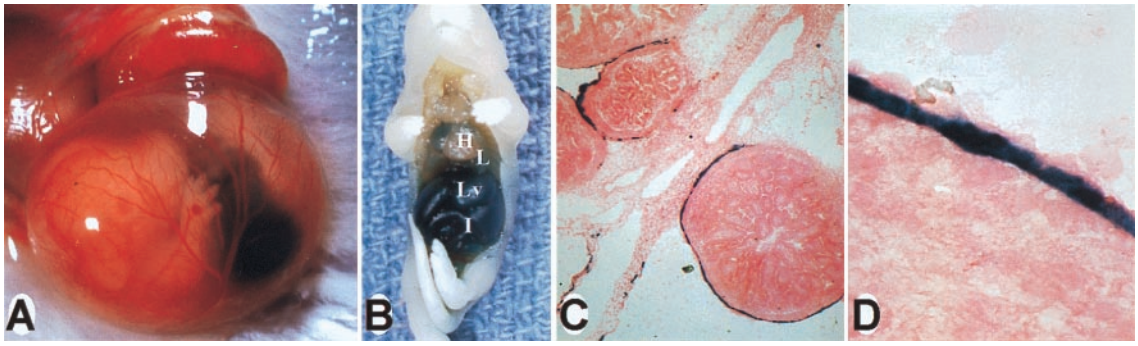

Fig. 1. The i.p. injection approach in a CD-1 mouse at E15. (*A*) Methylene blue (10 μl at 0.25%) was injected by a transuterine approach into the fetal abdomen, filling the i.p. cavity. The fetus, placenta, and intact amniotic sac were delivered through a hysterotomy. (*B* and *C*) Pattern of β-galactosidase (*LacZ*) expression in tissues at E18 from CD-1 fetuses injected with 1×10^7 plaque-forming units (pfu) of Ad-*LacZ* at E15. (*B*) X-Gal staining is present in the intestine (I), liver (Lv), peritoneum, heart (H), and lungs (L) at E18. (*C*) Tissue sections, counterstained with nuclear fast red, show *LacZ* staining of fetal peritoneum and intestinal serosa at E18 ($\times$50 magnification). (*D*) High-magnification photomicrograph of *LacZ*-expressing cells in the intestinal serosa (oil lens, $\times$1,000).

adenoviral transduction of fetal tissues. Fetuses were viewed under magnification during transuterine injection of 0.25% methylene blue dye into the fetal i.p. space. The dye diffused throughout the abdominal cavity and was limited to the subdiaphragmatic region (Fig. 1*A*). The sites of transferred gene expression were examined after *in utero* i.p. delivery of 1×10^7 pfu of Ad-*LacZ* in CD-1 fetuses at E15. Gross and microscopic examination of fetal tissues identified X-Gal staining of the peritoneum, intestinal serosa, and capsule of the liver, with occasional staining seen in the lungs and atria (Fig. 1 *B–D*). The distribution of *LacZ* expression in tissues from these E18 fetuses was comparable to that observed in previous studies establishing the hepatic tropism of adenoviral vectors (15, 23).

i.p. Delivery of Ad-Luciferase Produces High-Level Hepatic Luciferase Expression. All further studies were performed by E15 transuterine injection of carrier (X_HX) females mated with affected males. The expected genotypic frequency of pups from these crosses is 50% affected (hemophilic; X_HX_H or X_HY), 25% normal (XY), and 25% carrier (X_HX). Thus, the survival of hemophilic and normal fetuses could be compared within the same litter. To examine the reproducibility of transduction in fetuses from a single litter, luciferase expression was examined in the liver and lungs of 12 fetuses after i.p. injection of 1×10^7 pfu of an adenoviral vector containing the luciferase reporter gene (AdCa18Luc). All fetuses were alive at E18 as determined by the presence of cardiac activity and movement. The average luciferase activity was 50,624.07 $\pm$ 12,843.42 RLU/μg protein in the livers (Fig. 2*A*) and 8,480.28 $\pm$ 4,568.86 RLU/μg protein in the lungs (Fig. 2*B*). Pups in the litter were present with the predicted genotypic frequencies: three normal males (XY), three carrier females (X_HX), and six affected animals [X_HY (n = 5) and X_HX_H (n = 1)].

***In Utero* Adenoviral Delivery Does Not Affect Growth, Development, or Fertility of Pups.** In prior studies of *in utero* gene transfer, injection of fetuses with adenoviral vectors was associated with significant morbidity and mortality. To compare the toxicity of i.p. injection of adenoviral vectors with the morbidity of the injection procedure itself, the survival rates of hemophilic fetuses injected with 5–20 μl of NS and fetuses injected with recombinant adenovirus were determined. One litter was injected i.p. under each of six conditions: i.p. injection of 5 μl of NS (n = 9), 10 μl of NS (n = 10), 15 μl of NS (n = 8), 20 μl of NS (n = 7), and 1×10^7 pfu of AdCa18Luc in 5 μl of NS (n = 12). Mock injection by needle insertion only was performed in one litter (n = 10). At E18, 55 of 56 fetuses were viable as determined by the presence of cardiac activity and movement. Thus, the injection of increasing volumes up to 20 μl did not adversely affect survival in this group of animals.

To determine whether *in utero* adenoviral injection had deleterious effects on the development and growth of animals, pups injected *in utero* with AdCa18Luc were followed longitudinally. Pups were weighed on their day of birth and at 7, 14, 21, and 28 days of life. Animals had no differences in weight or abnormalities in physical development compared with age-matched controls observed over the same 28-day period (data not shown). Although injection of adenovirus has been associated previously with hepatotoxicity, we have not observed any elevation in hepatic transaminase levels in live-born pups 5 days after *in utero* injection of 1×10^7 pfu of AdCa18Luc (16). The frequency of live-born pups in litters injected with AdCa18Luc at E15 was 91% (20 of 22 fetuses injected). The frequency of live-born hemophilic animals (10 of 22) in these experiments was not reduced in comparison to the survival of normal and carrier siblings from the same mother. The fertility of females previously injected *in utero* with adenoviral vectors as well as the health of the litters they produce were comparable to those of uninjected females (G.S.L. and K.M.L.G., unpublished observations). Thus, i.p. injection of up to 1×10^7 pfu of recombinant adenovirus *in utero* was not associated with postnatal morbidity.

Therapeutic FVIII Levels Are Achieved After *in Utero* i.p. Injection of Ad-mFVIII. To follow the level and duration of mFVIII expression after injection of Ad-mFVIII *in utero*, seven carrier females

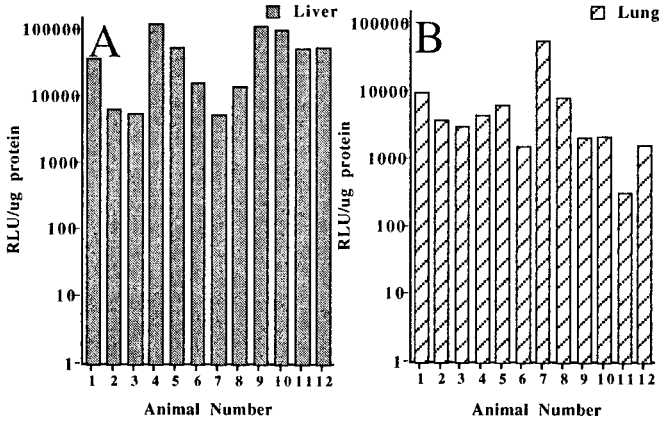

Fig. 2. Luciferase expression at E18 (after i.p. delivery of 1×10^7 pfu of AdCa18Luc on E15) in livers (*A*) and lungs (*B*) of fetuses from carrier females bred with hemophilic males. The luciferase activity in fetal organs from littermates is expressed as RLU/μg protein. Numbers on the *x* axis denote 12 individual fetuses within the litter.

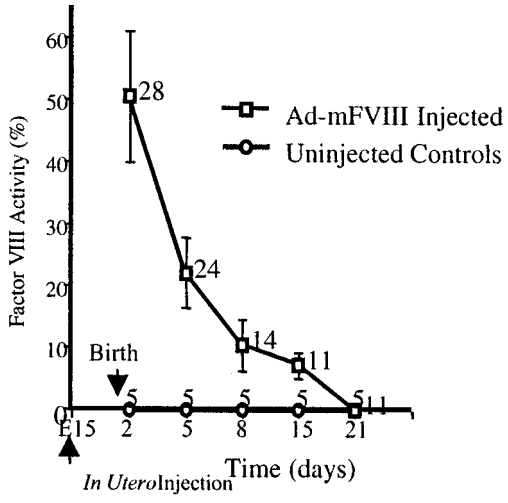

Fig. 3. Duration of FVIII expression in neonatal hemophilic mice after *in utero* i.p. injection of 3.3×10^5 pfu of Ad-mFVIII at E15. FVIII activity is expressed on the *y* axis as the percentage of normal mouse FVIII levels. The age of the mice is indicated on the *x* axis. The number of animals in each group is shown above each time point. Open squares, Ad-mFVIII-injected hemophilic animals; open circles, uninjected hemophilic animals. The Ad-mFVIII-injected group decreases in number, because some animals died during repeated plasma sampling procedures.

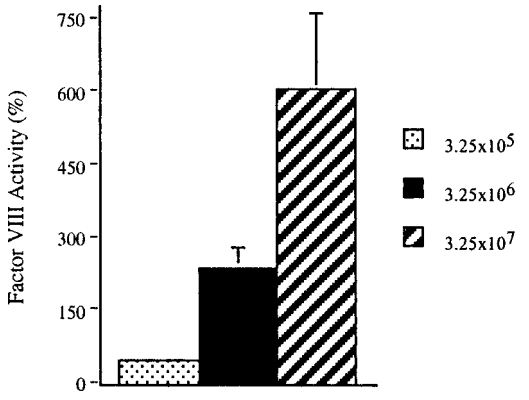

Fig. 4. Dose-response relationship between pfu of Ad-mFVIII delivered i.p. and level of FVIII activity achieved in hemophilic animals. The level of FVIII activity at day 2 of life is plotted on the *y* axis. Animals receiving 3.3×10^5 pfu of Ad-mFVIII are represented by the stippled bar; animals injected with 3.3×10^6 pfu of Ad-mFVIII are represented by the black bar; and the group injected with 3.3×10^7 pfu of Ad-mFVIII is represented by the striped bar. Each group included three or more animals. The line above each bar indicates SEM.

underwent transuterine i.p. injection with 3.3×10^5 pfu of Ad-mFVIII at E15. The live-born rate of injected pups was 87.2% ($n = 68$ of 78 injected). The genotypic distribution of pups was as expected: 47.8% affected (19 X_HY and 13 X_HX_H), 22.1% carrier ($n = 15$), and 30.1% normal ($n = 21$). Two hemophilic males died of unknown causes on day 1 of life. The remaining affected mice underwent serial plasma sampling and analysis of FVIII activity. Mice were tattooed for identification on the first day of life, and screening PCR was performed. Only affected newborns (X_HY and X_HX_H) were subjected to further analysis. FVIII levels in uninjected or saline-injected hemophilic animals were reproducibly <1% (Fig. 3). FVIII activity in plasma in 2-day-old mice after i.p. injection of Ad-mFVIII on E15 was 50.7 ± 10.5% (SEM) of normal levels ($n = 28$; Fig. 3). FVIII activity declined to 22.5 ± 5.7% by day 5 of life ($n = 14$), 10.4 ± 4.2% at day 8 of life ($n = 11$), and 7.2 ± 2.2% by day 15 ($n = 11$). There was no detectable FVIII activity in injected animals at day 21 of life ($n = 11$; Fig. 3). The relationship between the dose of Ad-mFVIII injected i.p. at E15 and the plasma level of mFVIII activity produced was analyzed in two additional litters. One litter was injected with 3.3×10^6 pfu of Ad-mFVIII ($n = 6$) and the other with 3.3×10^7 ($n = 6$) pfu. After injection of 3.3×10^6 pfu of Ad-mFVIII, circulating FVIII levels on day 2 of life were 239.3 ± 42.6%, whereas injection of a 10-fold higher dose (3.3×10^7) produced mFVIII levels of 609.0 ± 156.6% (Fig. 4). Thus, increasing doses of Ad-mFVIII produced plasma FVIII activity in the supranormal range.

Adenoviral DNA Is Found in Extraperitoneal Sites After *in Utero* i.p. Injection. The tissue distribution of adenoviral DNA in animals after *in utero* i.p. injection at E15 was analyzed in E18 fetal tissues from a litter injected with 3.3×10^5 pfu Ad-mFVIII and in tissues from 2-day-old pups previously injected with 3.3×10^7 pfu at E15. In these and other studies we have performed, the injection of adenoviral vector was not associated with increased mortality (23). Viral genome copy number was estimated by semiquantitative PCR and comparison with standard DNA samples with known quantities of adenoviral DNA as described above. Negative control samples included genomic DNA from uninjected carrier (X_HX) females and from animals injected with an unrelated adenoviral vector (AdCa18Luc; Fig. 5). Animals injected with 3.3×10^5 pfu at E15 had high levels of viral DNA in the liver, intestine, and spleen at E18 (Table 1). Adenoviral sequences were not detected after amplification of DNA from the brain, heart, lung, and thymus from fetuses injected with 3.3×10^5 pfu (limit of detection: 1 viral genome per 5,830 cells). In 2-day-old pups injected at E15 with the highest dose (3.3×10^7 pfu) of Ad-mFVIII, viral DNA was detected in tissues distant from the i.p. space (Fig. 5), including the brain, heart, and thymus (Table 1). Thus, at larger doses of adenovirus, Ad-mFVIII appears in the systemic circulation and in tissues distant from the site of injection.

The possibility of germ-line integration is an important concern for any future clinical application of *in utero* gene transfer. Although it is difficult to exclude rare integration events, PCR analysis of genomic DNA from the total gonadal tissues of 9-week-old male and female mice previously injected *in utero* with 3.3×10^7 pfu of Ad-mFVIII did not detect viral sequences (data not shown). In addition, amplification of DNA from the livers of F_1 progeny from the above animals also failed to detect Ad-mFVIII DNA (data not shown).

Discussion

We have used efficient recombinant adenoviral vectors to achieve reproducible gene transfer after *in utero* i.p. injection in a murine model, without significant morbidity or mortality. Injection of Ad-mFVIII in FVIII-deficient fetuses produced dose-related therapeutic levels of circulating FVIII and phenotypic correction of hemophilia A throughout the neonatal period. Although *in vivo* gene therapy studies have largely focused on gene delivery in adult animals, the use of *in vivo* targeting of fetal tissues has several important advantages. For many genetic disorders, the affected tissue cannot be transplanted readily, and the ability to intervene therapeutically, earlier in development would reduce or eliminate morbidity and mortality later in life. *In utero* gene delivery and persistent gene expression may induce tolerance to therapeutic proteins that could elicit toxic or inactivating immune responses when administered postnatally. Finally, the development of strategies for effective *in utero* gene transfer in fetuses creates the possibility of targeting expanding stem cell populations that may be absent or quiescent in adult tissues. Although the use of nonreplicating, episomal adenoviral vectors precludes long-term analysis of gene transfer in self-

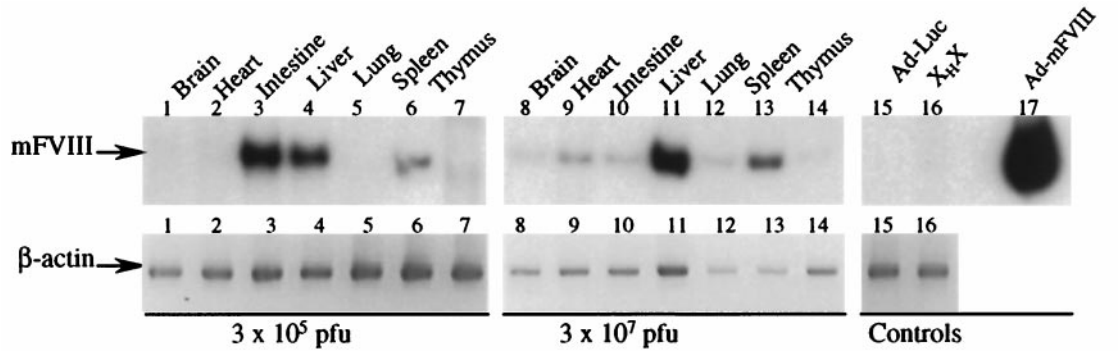

Fig. 5. Copy number and tissue distribution of Ad-mFVIII genomes as determined by semiquantitative PCR. (*Upper*) Autoradiographs of PCR products after amplification of genomic DNA with Ad-mFVIII-specific primers are shown. The tissues analyzed are indicated above each lane. The predicted PCR product is 597 bp in length. Negative control samples include DNA from uninjected carrier females (X_HX) and DNA from an adult mouse injected i.v. with AdCa18Luc. As a positive control for Ad-mFVIII PCR, hepatic DNA from an adult hemophilic animal injected i.v. with Ad-mFVIII was amplified and is shown in lane 17. (*Lower*) PCR amplification with mouse β-actin-specific primers of the same DNA samples shown above. The dose of Ad-mFVIII previously delivered i.p. in each group of samples appears at the bottom of the figure.

renewing progenitor cells in these studies, future *in utero* gene transfer studies with integrating or self-replicating vectors will make such studies feasible.

Previous studies of *in utero* gene transfer have focused on delivery of viral vectors by fetal amniotic fluid instillation, direct intratracheal injection (10–12), and placental transduction (24). However, this report concentrates on i.p. *in utero* gene transfer achieving short-term phenotypic correction in a murine model that reproduces a human disorder. At an i.p. dose of 3.3×10^5 pfu Ad-mFVIII at E15, PCR analysis identified viral genomes in the liver, intestine, and spleen. i.p. delivery in the fetus resulted in reproducible high-level expression in the liver, consistent with the hepatic tropism of adenovirus (15, 16). When higher doses of adenovirus (3.3×10^7 pfu) were delivered, transduction in distal tissues including the brain, heart, intestine, liver, lung, spleen, and thymus was observed, with the highest copy number in the liver.

There are several possible routes by which adenovirus injected into the peritoneal cavity eventually transduces hepatic and other cells. These include (*i*) direct uptake into lymphatic and blood vessels in the peritoneum, (*ii*) inadvertent injection into the systemic circulation, and (*iii*) passage through 4- to 12-μm intermesothelial cell stomata in the diaphragmatic peritoneum (22). Adenoviral particles (70 nm in diameter) may pass through these stomata and eventually gain access to the systemic circulation. In addition, there may be direct uptake of adenoviral particles into endothelial cells in blood and lymph vessels in the peritoneum and other tissues. It is likely the Ad-mFVIII DNA we detected in many tissues is due in part to the transduction of vascular endothelium in these organs (16, 23).

We have shown that delivery of 3.3×10^5 pfu of Ad-mFVIII *in utero* produces therapeutic levels of FVIII for the first 2 weeks of life, despite the dilution of adenoviral genomes in the growing animals. This dose represents a 10- to 100-fold lower dose of adenovirus than has routinely been delivered in adult mice (1×10^6 pfu/g in our studies vs. 3×10^7 pfu/g or 1×10^9 pfu/30 g for adult mice; ref. 25). Two processes generally limit the duration of replication-deficient adenoviral-mediated expression in adult immunocompetent animals. First, the nonreplicating vector remains episomally located and is progressively lost on cellular division. This process is particularly relevant in the rapidly growing fetus. Second, humoral and cytotoxic T lymphocyte responses directed against late viral proteins result in elimination of transduced cells, hepatocellular damage, and a concomitant elevation in the serum alanine aminotransferase levels (16, 26). However, several observations suggest that these immune responses may not play an important role after adenoviral injection *in utero*. First, in our previous studies, the serum alanine aminotransferase levels in neonatal mice injected with comparable doses of adenoviral vectors *in utero* were not significantly different from those observed in age-matched uninjected control pups (16). Furthermore, in preliminary studies, we have been able to reinject animals previously injected *in utero* with adenoviral and nonviral vectors and to reexpress the transgenes at high levels. Similar results have been reported in newborn animals injected with adenoviral vectors in the neonatal period and again as adults (27). This efficient reexpression of transferred genes may be attributed to a lack of immune priming because of the immaturity of the murine immune system and of T cell responses during late gestation and in the neonatal period.

This study shows the potential for fetal gene therapy to ameliorate and even correct congenital disorders during the perinatal period. Gene expression *in utero* that is either short-term and stage-specific or more durable may be useful in different settings. For example, stage-specific gene expression may be effective for rescue of lethal knockout models or in the acute surgical setting for patients with bleeding disorders. Recent studies with a decoy antisense RNA construct established

Table 1. Average adenoviral genome copy number in tissues

Dose of Ad-mFVIII, pfu	Number of Ad-mFVIII DNA per 1,000 cells						
	Brain	Heart	Intestine	Liver	Lung	Spleen	Thymus
3.3×10^5	ND	ND	57.6	43.8	ND	19.7	ND
3.3×10^7	6.6	26.0	13.4	227.0	8.4	63.9	0.9

Litters of carrier females (X_HX) bred with hemophilic males (X_HY) were injected at E15 with either 3.3×10^5 pfu of Ad-mFVIII or 3.3×10^7 pfu. Fetuses injected with 3.3×10^5 pfu were analyzed at E18, whereas fetuses with 3.3×10^7 pfu were analyzed on the 2nd day of life. ND, not detectable.

the efficacy of short-term gene transfer and expression in maintaining patency of the ductus arteriosus (28). In the case of hemophilia A, it may be possible to decrease the risk of catastrophic bleeding in severely affected infants during labor and delivery by introducing a gene therapy vector *in utero* (29, 30). Our results suggest that even fragile murine knockout models, such as the FVIII exon-17-disrupted animals that we used in these studies, will be useful for developing prenatal and postnatal approaches for gene delivery.

We thank Dr. Y. W. Kan for his ongoing interest in this project. This work was supported in part by National Institutes of Health Grant HL53762 (to K.M.L.G.) and a scholarship from the American College of Surgeons (to G.S.L.).

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