In vivo hepatic gene therapy: Complete albeit transient correction of factor IX deficiency in hemophilia B dogs

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Contributed by Kenneth M. Brinkhouse, November 23, 1993

ABSTRACT Hemophilia B is a bleeding disorder caused by mutations in the factor IX gene. The disorder is X-linked recessive with a prevalence of about 1 in 30,000 Caucasian males. Factor IX is naturally synthesized in the liver and secreted into blood. Here we report the construction of recombinant adeno viral vectors containing the canine factor IX cDNA that are capable of transducing hepatocytes in mice at high efficiencies in vivo without partial hepatectomy. The recombinant viral vector was used to treat hemophilia B dogs by direct vector infusion into the portal vasculature of deficient animals. Plasma factor IX concentrations in the treated hemophilia B dogs increased from 0 to 300% of the level present in normal dogs, resulting in complete amelioration of the disease as demonstrated by normal blood coagulation and hemostatic measurements. Although plasma factor IX concentration started to decline after a few days, therapeutic levels of factor IX persisted for 1–2 months in the treated animals. These results validate the principle of in vivo hepatic gene delivery to reconstitute the genetic deficiency in a large animal model and suggest that gene therapy is achievable when long-acting vectors are developed.

Hemophilia B is an X-linked disorder resulting in a deficiency of plasma factor IX, which is normally synthesized and secreted from the liver. The disease affects 1 in 30,000 males (1). Various human protein replacement therapies have significantly improved the clinical outcome of affected individuals, yet there still exists a high degree of morbidity and mortality in part due to previous contamination with human immunodeficiency virus and other hepatitis viruses. The current expense of virus-free factor IX usually limits therapy to treatment of bleeding episodes once they occur. Due to the relatively short half-life of factor IX in the circulation, affected individuals have life-long risks of central nervous system bleeds, chronic arthropathies, and other life-threatening hemorrhage. Replacement therapy has improved, but has failed to abrogate, chronic arthropathy, particularly in severely affected patients. A number of cell types in different organs have been targeted for somatic gene therapy of hemophilia B, which include fibroblasts, myoblasts, endothelial cells, keratinocytes, and hepatocytes (2–8). We have pursued the liver as the target organ because hepatocytes represent the natural site of factor IX synthesis and secretion. We have previously been able to transduce hepatocytes in hemophilia B dogs after partial hepatectomy using a retroviral vector containing the factor IX cDNA (9). This resulted in long-term expression of about 0.1% of the normal plasma factor IX concentration. To attempt to produce greater levels of recombinant factor IX in animals, we have pursued replication-deficient recombinant adenovirus (10–12) as a gene delivery vector for the treatment of hemophilia B.

METHODS

Canine Factor IX Adenoviral Vectors. The isolation of a functional canine factor IX cDNA has been described (9). This cDNA was cloned into a Bluescript vector containing either the 540-bp phosphoglycerokinase promoter (13) or the Rous sarcoma virus (RSV) long terminal repeat promoter and the 280-bp bovine growth hormone polyadenylation site. The expression cassette was cloned into a derivative of the PXC2 plasmid (14), PXCJL1 (a gift from Frank Graham), and cotransfected with the pJM17 plasmid (15) into 293 cells (16). Recombinant adenoviral plaques were isolated as described (15) and analyzed for canine factor IX protein production in vitro. The recombinant viral particles were grown and purified as described (11) except that after double CsCl banding the virus was extensively dialyzed against 10 mM Tris/1 mM MgCl₂, pH 7.4/10% glycerol. The purified virus was stored in aliquots at –80°C. The virus titer was determined by OD measurements and plaque assay and ranged from 1–3 × 10¹¹ plaque-forming units (pfu) per ml.

Animal Studies. All the animal studies were performed in accordance with the institutional guidelines at the Baylor College of Medicine and the University of North Carolina. Hemophilia B dogs were from the Chapel Hill inbred strain (17). Direct in vivo adenoviral-mediated gene transfer into hepatocytes of C57BL/6 mice was as described (10). The placement of an infusion port-catheter into the splenic vein of dogs was described (9). Hemostatic coverage was maintained with multiple infusions of fresh frozen normal dog plasma given immediately before and for 2–5 days after the operation (41.4–68.2 units of factor IX per kg). To demonstrate that the exogenous factor IX had decayed to presurgical levels, the plasma factor IX concentrations were monitored by bioassay and whole-blood clotting times (WBCTs) prior to adenoviral administration (at least 2 weeks). Purified adeno virus was diluted with about 2 volumes of sterile DMEM-H medium and given in two infusions via the infusion port; each infusion lasted about 15–25 min, with a 15-min pause between infusions. The total volume infused was about 35–40 ml.

Hemostatic Parameters After Adenoviral Gene Therapy. The WBCTs, factor IX bioassay, and nonactivated partial thromboplastin time (PTT) were performed as described (18–20). A modification of the method of Jim (21) was used for the thrombin clotting time (TCT) test. The ELISA immunoassay (8) for quantitation of factor IX antigen was performed by

Abbreviations: RSV, Rous sarcoma virus; PTT, partial thromboplastin time; WBCT, whole-blood clotting time; TCT, thrombin clotting time; pfu, plaque-forming units.

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using a polyclonal rabbit anti-dog factor IX antibody, which has no cross-reactivity with mouse factor IX. The primary antibody was diluted 1:200, and the same antibody was conjugated to horseradish peroxidase as per the manufacturer’s specification (Pierce) and used at a 1:25 dilution as the secondary antibody. Pooled normal dog plasma was considered to contain 11.5 µg of canine factor IX per ml and was used as a standard. The sensitivity of this assay was to 1 ng/ml (9). The secondary bleeding time (22) was carried out as described.

PCR Quantification of Adenoviral DNA in Tissues. One microgram of DNA was used per 50-µl reaction mixture. Experimental DNA was diluted by 10-fold serial dilutions from 1 µg to 1 ng. Two pairs of primers were used. The first was in the RSV promoter (5'-GTAAGGTGTAGATGCCTTGCCT-3'), and the second was in the canine factor IX cDNA (5'-TGCAGTTACCACCCATTTTTCAT-3'), giving rise to a specific 1-kb product. The second pair of primers were in the RSV promoter (5'-GACTCCTAACCGCGTACA-3') and the adenoviral genome (5'-CTCAATCTGTATCTTCATGC-3'), giving rise to a 280-bp product. Control reactions containing DNA from 1 to 10² viral particle were run in parallel. After 30 cycles of amplification, 10 µl was applied to a 2% agarose gel. The intensities of the bands in the linear region were compared to calculate the number of viral particles per microgram of genomic DNA. From this, the number of adenoviral genome copies per diploid cell was estimated.

RESULTS
Recombinant adenoviral (Ad) vectors were constructed that contain the canine factor IX (cFIX) cDNA under the transcriptional control of the phosphoglycerokinase promoter (PGK) or RSV long terminal repeat promoter (Fig. 1A); these vectors are referred to as Ad/PGK-cFIX and Ad/RSV-cFIX, respectively. The vectors were shown to transduce cells and produce canine factor IX in cultured fibroblasts prior to their use in animals (data not shown). We have previously determined that 10¹⁰ recombinant adenoviral particles can be infused into the portal vein of a mouse, which resulted in 100% hepatocyte transduction without significant pathologic changes (10). To test the cFIX vectors for their expression by transduced hepatocytes in vivo, 10¹⁰ purified viral particles were infused into the portal vasculature of mice. The animals were periodically bled and serum factor IX antigen concentrations were determined by ELISA (Fig. 1B). The use of the Ad/RSV-cFIX vector resulted in persistent expression for 2–3 months at a serum level that was 10- to 20-fold greater than the animals infused with the Ad/PGK-cFIX. Thus the former vector was used to treat hemophilia B dogs.

To determine whether dog hepatocytes could be transduced with adenoviral vectors in vivo, the Ad/RSV-Bgal vector (23) was infused into the portal vasculature of normal dogs via a subcutaneous port with catheter tip placed in the splenic vein close to its junction with the portal vein (9). After 8 days of viral transduction the animals were sacrificed, and tissues and hepatocytes were obtained for 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) staining. The cultured cells were stained with X-gal within 1 hr of plating. The transduction efficiency was determined by counting the proportion of blue cells, which ranged from about 20% to 50% between different animals. X-gal-stained histochemical liver sections showed similar results (data not shown). Routine

![Fig. 1](image-url)

**Fig. 1.** Construction of adenoviral vectors containing the canine factor IX cDNA and direct hepatic gene transfer in mice. (A) The DNA structure of the Ad/PGK-cFIX and Ad/RSV-cFIX adenoviral vectors. The shaded areas represent viral sequences. PGK, phosphoglycerokinase promoter; bpA, bovine polyadenylation signal. (B) Serum canine factor IX antigen concentrations in mice after adenoviral-mediated hepatic gene transfer. Recombinant adenovirus (1 × 10¹⁰ pfu) particles were infused into the portal vein of C57BL/6. Periodic blood samples were obtained for canine factor IX analysis by ELISA. Each line represents an individual animal. The open circles represent animals infused with the Ad/PGK-cFIX vector, and the closed circles represent the animals infused with the Ad/RSV-cFIX vector. Control animals infused with the same quantity of control adenoviral vectors had no detectable serum canine factor IX antigen (not shown).
histological sections of the liver were normal. Occasional blue cells were found in the spleen but not in other tissues.

To determine whether the Ad/RSV-cFIX vector would correct the coagulation defect in animals, the recombinant vector was injected into the portal vasculature 1 month after the installation of infusion ports in two factor IX-deficient dogs from the Chapel Hill inbred strain (17). The missense mutation found in these animals results in the absence of detectable factor IX in the plasma (24). Transient pallor occurred during the infusion, and transient temperature elevation occurred immediately postinfusion. The temperature elevation was self-limited and resolved within 8–12 hours after treatment. It is not known if this was the result of the adenovirus or a copurifying contaminant.

Plasma was obtained periodically for canine factor IX measurements by immunologic and bioassays. Hemostatic parameters were monitored by changes in the WBCTs and PTTs. The factor IX concentrations in dogs 1 and 2 reached supraphysiologic values during the first 4 days after adenoviral vector infusion: dog 2 had 300% of the normal plasma level on day 1, whereas dog 1 had 250% of the normal plasma level on day 2 (Fig. 2A). The plasma factor IX levels started to decline, and by 3 weeks, levels were about 1% of the average in normal dogs; by two months they were around 0.1% of normal and became undetectable in one of the treated animals after 100 days. The PTT, a measure of the intrinsic clotting mechanism, rapidly shortened from 174–196 sec to 47–64 sec by the fifth day (normal range, 42–47 sec) (Fig. 2B). The WBCTs in the treated animals became normal or near normal 1–8 days after therapy began (Fig. 2C). Thereafter, both the PTTs and WBCTs slowly increased, which is consistent with the gradual decline in plasma factor IX levels.

Because hemophilia B dogs 1 and 2 had therapeutic factor IX levels within 24 hr of infusion, a third dog was infused with the Ad/RSV-cFIX adenoviral vector, and plasma was sampled every 4–6 hr (Fig. 2D). Normal levels of factor IX were achieved by 18 hr, and the peak concentration was obtained at 48 hr, before the slow decline started. In this animal, two additional tests were performed prior to and after treatment, TCT and the secondary bleeding time. The TCT was measured to estimate the fibrinogen concentration because of the transient pallor and febrile reaction the dogs experienced with adenoviral gene therapy. The TCT is normal in hemophilia B dogs; however, it was transiently prolonged after adenoviral administration. The TCT value at 6 hr was 15.6 sec, with the greatest prolongation of 22.2 sec at 12 hr, and a return to the normal value of 12.1 sec at 96 hr. These data suggest the presence of a transient hypofibrinogenemia. For the secondary bleeding time, the pretreatment value was 14.5 min. On days 1–8 posttreatment, the values were in the range of 1.25–1.75 min, which is normal. Thus, the recombinant factor IX was able to correct the hemophilic animal’s response to a hemorrhagic challenge.

The Ad/RSV-Bgal studies suggest that after portal vein infusion the majority of virus results in transduction of liver, with small amounts of the virus infecting other tissues. To quantitate this, a similar dose of Ad/RSV-cFIX virus used to treat the hemophilia B dogs was infused into a female carrier. The hemophilia B heterozygote had a baseline value of 5200 ng of factor IX per ml of plasma; the factor IX level was raised to 33,000 ng/ml on the third day. The animal was sacrificed at this time, and genomic DNA was extracted to determine the relative distribution of the recombinant adenoviral genome in tissues by a semiquantitative PCR assay (Table 1).
The amount of adenoviral DNA per cell averaged 0.7-7 copies per hepatocyte, which is at least 10-fold greater than the next abundant tissues, which included the spleen and stomach. The presence of some adenovirus in these tissues is not unexpected because of the vascular tributaries of the splenic vein from these organs. Other organs listed in the table had significantly less adenoviral DNA.

At the time of sacrifice, tissues were also prepared for routine histological analysis. The results showed some mild degeneration and necrosis that was most apparent in the right lateral lobe of the liver. Transient mild hepatic necrosis has been observed in mice transduced with adenoviral vectors (10). It is of interest to note that in the Ad/RSV-Bgal-infused dogs, there was no apparent liver pathology 8 days after viral infusion. Other organs that were studied histologically included the kidney, thymus, lymph nodes, duodenum, spleen, brain, stomach, colon, heart, lung, and thyroid, which were all normal.

**DISCUSSION**

Recombinant adenoviral vectors have been used to transfer genes into a number of cell targets. We and others have used these vectors for the direct *in vivo* transfer of genes into the liver of animals (10-12). The study here demonstrates that recombinant adenoviral vectors can be used in a large animal model to completely ameliorate the coagulation defect in hemophilia B that results from the synthesis of high levels of factor IX in the liver. We have demonstrated in a rodent model that the degree of hepatic transduction is directly related to the dose of adenovirus infused. Because the amount of plasma factor IX produced is greater than the normal levels, less adenovirus will be needed to achieve the wild-type plasma concentration. In fact, considerably smaller doses will be needed to achieve 25% reconstitution, which is considered curative. One animal studied did have mild liver necrosis with adenoviral infusion. Transient mild hepatic necrosis has been demonstrated in the mouse in a dose-dependent manner (10). Thus, it is likely that the lower dose of adenovirus required for curative levels of factor IX will not result in hepatic toxicity.

The animals treated with adenovirus demonstrated a lag from the period of normal factor IX concentrations and normalization of the PTT. This in combination with the transient febrile reaction led us to consider the possibility of a mild disseminated intravascular coagulation event. This hypothesis is supported by the transient hypofibrinogenemia demonstrated in dog 3. Whether this event is a direct cause of adenovirus or a copurifying contaminant is not known. We have previously demonstrated a similar clinical response to a challenge of minute quantities of fetal calf serum (25), which is used to propagate the adenovirus, but until further studies are carried out, the cause of the transient clinical reaction and hypofibrinogenemia cannot be determined. It is likely that the reaction will be less significant if lower doses of adenovirus are administered to these animals.

Expression after adenoviral-mediated gene transfer in the hemophilia B dogs is transient and most likely results from the loss of DNA from transduced cells as we have demonstrated in the mouse (10). Interestingly, factor IX expression persisted longer in the mouse than the dog for reasons that are unclear at this time, although the canine factor IX concentrations did eventually begin to fall in the mouse. The kinetics of factor IX expression is different in mice infused with the Ad/PGK-cFIX vector than with the Ad/RSV-cFIX vector. The former vector results in peak expression within the first week followed by a slow decline. The mice transduced with the Ad/RSV-cFIX vector reach peak serum factor IX concentrations between 2 and 4 weeks after infusion before the concentrations begin to decline. In contrast, when this vector was used in the hemophilia B dogs, the maximal concentrations of plasma factor IX were detected within the first 2 days. The differences in expression may in part be due to differences in gene transcription from this promoter in the two species. The fall in serum factor IX in mice is consistent with the decline in the percentage of β-galactosidase-positive hepatocytes that were found at various times after hepatic Ad/RSV-Bgal administration in our earlier studies (10).

The nature of adenoviral DNA loss from transduced cells is currently not known. It is possible that transduced cells are slowly replaced or that the episonal DNA is slowly degraded in hepatocytes. We have attempted to infuse a second dose of recombinant adenovirus into the hemophilia B animal after recombinant factor IX was no longer detectable. No recombinant factor IX was present in the plasma after reinfusion, suggesting an immunologic block to reinfusion (data not shown). Antibodies to fiber are believed to be neutralizing, and the presence of antibodies may be responsible for the inability to reinfuse the animals and get secondary gene transfer. Additional studies will be necessary to define an immunologic block and to establish a causal relationship with the inability to give repeated doses for the purposes of hepatic gene therapy for hemophilia B.

The high efficiency of gene transfer and expression into the liver, albeit transient, demonstrates that *in vivo* gene delivery to the liver can be practical in humans in the future. Many metabolic disorders can be improved with even transient gene expression *in vivo*. This makes the recombinant adenoviral vector system an attractive one for future hepatic gene transfer and therapy, particularly if the lack of persistence can be addressed by further vector development.

We thank Robin Raymer and Pam McElveen (Chapel Hill, NC) for their expert technical assistance. This work was supported in part by National Institutes of Health Grants DK 44080 (Baylor College of Medicine) and HL 01648-46 and HL 26309-12 (University of North Carolina, Chapel Hill). M.A.K. is a recipient of an individual National Research Service Award Fellowship Award GM 13894, and S.L.C.W. is an Investigator of the Howard Hughes Medical Institute.


Table 1. Tissue distribution of adenoviral DNA in a hemophilia B heterozygote

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Copies per μg of DNA</th>
<th>Copies per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>10^5-10^6</td>
<td>0.7-7</td>
</tr>
<tr>
<td>Spleen</td>
<td>10^5-10^6</td>
<td>0.07-0.7</td>
</tr>
<tr>
<td>Stomach</td>
<td>10^5-10^6</td>
<td>0.07-0.7</td>
</tr>
<tr>
<td>Lung</td>
<td>10^5-10^6</td>
<td>0.007-0.07</td>
</tr>
<tr>
<td>Kidney</td>
<td>10^5-10^6</td>
<td>0.007-0.07</td>
</tr>
<tr>
<td>Heart</td>
<td>10^5-10^6</td>
<td>0.007-0.07</td>
</tr>
<tr>
<td>Ovary</td>
<td>10^5-10^6</td>
<td>0.007-0.07</td>
</tr>
<tr>
<td>Muscle</td>
<td>10^5-10^6</td>
<td>0.007-0.07</td>
</tr>
<tr>
<td>Lymph node</td>
<td>10^5-10^6</td>
<td>0.0007-0.0007</td>
</tr>
<tr>
<td>Brain</td>
<td>10^5-10^6</td>
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A 4.5-month-old factor IX-deficient heterozygous female dog (14 kg) was infused with 3 x 10^12 Ad/RSV-cFIX adenoviral particles via the portal vein. Peak factor IX plasma levels were similar to those obtained for dogs 1-3. Three days later the animal was sacrificed, and tissues obtained for DNA preparation and a semiquantitative PCR assay were used to estimate the amount of recombinant adenoviral DNA in each tissue.