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Direct intramuscular injection with recombinant AAV vectors results in sustained expression in a dog model of hemophilia

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A recombinant adeno-associated virus (rAAV) vector carrying the human factor IX cDNA was tested for safety and therapeutic gene expression in a canine model of human hemophilia B. Intramuscular delivery of rAAV was chosen based on our previous work which described long-term (>1.5 years) reporter gene expression in immunocompetent mice following direct muscle injection. For the current study, rAAV with the human factor IX (hF.IX) cDNA under the control of the cytomegalovirus (CMV) immediate–early promoter was constructed, and rAAV/hF.IX proved capable of transducing hemophilic dog primary fibroblast cultures in a dose-dependent fashion. Direct intramuscular injection of 2.5×10^{12} rAAV/hF.IX virus particles into the hindlimbs of a hemophilia B dog was tolerated without bleeding or systemic reaction, and the animal was asymptomatic throughout the entire study. Transient reduction in the whole blood clotting time (WBCT) occurred during the first week, with the anticipated development of an antihuman F.IX inhibitor antibody which corresponded with the loss of coagulation correction. At 10 weeks after vector administration, immunohistochemical analysis of injected muscle confirmed continued hF.IX expression. Limited areas of focal lymphocytic infiltration and myofiber pathology were detected which directly correlated with positive antibody staining for helper adenovirus contamination. PCR tissue analysis revealed rAAV/hF.IX DNA solely in injected muscle tissue and adjacent lymph node, without dissemination to other organs (including gonads). This first large animal study suggests that intramuscular gene delivery using rAAV vectors is safe and supports continued development of this approach for gene therapy of human diseases, including hemophilia B.

Keywords: *parvovirus; gene transfer; muscle; factor IX; canine; hemophilia B*

Introduction

Hemophilia B is a sex-linked, congenital bleeding disorder caused by deficiency of coagulation factor IX activity.[1] The clinical severity of bleeding closely corresponds to factor IX activity levels; factor IX levels less than 1% of normal cause severe disease (frequent spontaneous bleeding, especially in joints and soft tissues). While circulating levels of the factor IX protein normally equal 5 μg/ml, trace amounts of F.IX support coagulation and it can be expected that levels of 1–5% of normal would significantly ameliorate the chronic complications of the bleeding phenotype.

Gene therapy offers an attractive approach by which the goal of prophylactic hemostasis may be achieved without the extreme costs, infectious and thrombotic risks, and need for frequent administration associated with repeated injections of factor IX concentrates. The adeno-associated virus (AAV), in particular, appears to offer several advantages as a F.IX gene transfer vector. These include: (1) the safety of a nonpathogenic virus, which also does not require tissue-injuring pretreatment or expression of viral proteins to achieve persistence; (2) the capacity for gene delivery into terminally differentiated, nondividing and dividing cells; and (3) the potential for sustained expression of transgenes following conversion from input recombinant viral DNA to high molecular weight DNA form.[2,3]

Several tissues have been targeted for factor IX gene transfer, including skin fibroblasts, keratinocytes, hepatocytes and capillary endothelial cells.[4] In addition, skeletal muscle tissue has emerged as an important target for gene delivery. The ability of myoblasts, in particular, not only to transcribe the factor IX transgene but also to perform the series of post-translational modifications required for functional activity has been established.[5,6] Genetically modified muscle cells are capable of producing a range of secreted proteins, including human growth hormone, erythropoietin, alpha-1-antitrypsin, and interleukin-10.[7–10] Recently, Xiao *et al*[3] demonstrated *in vivo* that mouse skeletal muscle, transduced with an *E. coli* β-galactosidase gene in a recombinant AAV (rAAV) vector, expressed the transgene for greater than 1.5 years

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in immunocompetent animals; Southern analysis of genomic DNA from transduced muscle suggested the expression persisted via high molecular weight viral genomes. More importantly, these animals did not mount a cellular immune response against the rAAV-transduced cells. These results have subsequently been reproduced by other investigators.[11,12] In addition, intramuscular delivery of the human erythropoietin cDNA using rAAV produced systemic erythropoietin production for up to 40 weeks in mice. Using the same dose, a 50-fold greater expression following i.m. injection *versus* i.v. injection was observed, supporting this tissue as a preferred target for rAAV gene transfer.[8] The sustained expression of nonmurine proteins in these mouse models following rAAV-mediated intramuscular delivery suggested a similar approach for human F.IX delivery.

Targeted delivery to the muscle using rAAV in a large animal model of hemophilia B is a first step towards a clinical gene therapy trial. The Chapel Hill strain of hemophilia B dogs are a well-described hemophilia model, in which a complete absence of circulating factor IX results in a phenotype typified by recurrent spontaneous hemorrhage. The canine protein shares 85% homology with human protein, and infusion of human recombinant or plasma-derived factor IX in the dog model results in correction of blood clotting.[13,14] We report here the first study demonstrating sustained expression of human factor IX in the muscle of a hemophilia B dog following rAAV vector transduction.

Results

Transduction of primary hemophilic dog fibroblasts with rAAV/h.FIX

To test rAAV/hF.IX gene transfer and human factor IX expression in cells from the canine hemophilia B model, the human factor IX cDNA, under the control of the cytomegalovirus immediate–early promoter, was inserted into an AAV vector (Figure 1). The hF.IX cDNA contained a portion of the first intron of the factor IX gene, previously reported to increase mRNA stability,[15] and the first 50 nucleotides of 3′ untranslated region. The CMV promoter element has been previously shown to have sustained activity in muscle after intramuscular administration of an AAV β-galactosidase-expressing vector.[3] The final CMV promoter/factor IX expression cassette was built into an AAV vector, and designated pTRhFIXm1. This AAV vector consists of 145 bp AAV inverted terminal repeat (ITR) sequences, which are the only required elements for packaging and integration of the AAV vector,[2] flanking the hFIX gene cassette. Before producing recombinant virus, the plasmid was tested in a transient expression assay for expression of human factor IX protein using calcium-phosphate transfection of human embryonic kidney 293 cells and a F.IX ELISA assay. The medium overlying transfected 293 cells was also assayed for functional activity in a standard activated partial thromboplastin time assay (APTT), and demonstrated procoagulant activity corroborating the antigen results detected by ELISA (data not shown).

rAAV/hF.IX was tested for transgene expression by infection of primary fibroblasts isolated from one of the Chapel Hill strain of hemophilia B dogs. This strain of dogs makes no detectable canine F.IX and therefore should not confound our assay results. Early passage fibroblasts were plated at an initial concentration of 10^5 cells per well in six-well tissue culture plates and allowed to grow following infection with recombinant rAAV/hF.IX. The virus titer of the rAAV stock was 1.5×10^{12} total virus particles/ml and cells were infected at doses ranging from zero to 5×10^5 rAAV particles per cell. Expression of human factor IX into the media overlying 10^5 infected cells per 24-h period was assayed by ELISA. Secretion of factor IX was undetectable in tissue culture media on the first day after transduction in all conditions, but increased steadily over 1 week, from 0 to 350 ng FIX/24 h/10^5 cells plated at the optimal dose (1×10^5 particles per cell). Human factor IX protein expression increased as a function of virus dose up to 1×10^5 (10^3 particles per cell yielding 12 ng, 10^4 particles per cell 190 ng, 10^5 particles per cell 350 ng FIX/24 h/10^5 cells plated) before growth arrest of the primary cells at the end of the second week.

Transgene expression in vivo in hemophilia B canine model

Sera from six hemophilia B dogs were screened and found to be negative for neutralizing antibodies capable of interfering with rAAV infection and transduction *in vitro* (data not shown). rAAV/hF.IX isolated from the peak fraction of a $CsCl_2$ density gradient preparation of virus was injected intramuscularly into the hindlimbs of one of the screened

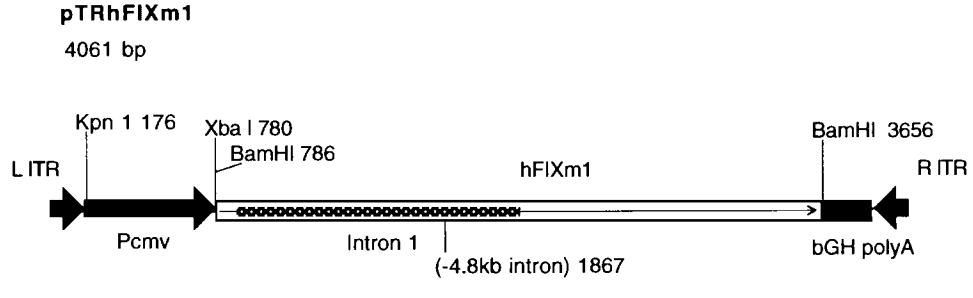

Figure 1 *Recombinant AAV-factor IX expression construct pTRhFIXm1. The human factor IX cDNA was cloned directionally downstream of the CMV promoter in the vector pTR-UF2. The hFIX cDNA, derived from p416FIXm1, includes 1.4 kb of hFIX first intron, with 4.8 of the first intron removed at the site noted. Of the backbone plasmid pTR-UF2, only the promoter and polyadenylation signals are packaged with the factor IX transgene into the recombinant virus, flanked by the AAV inverted terminal repeat structures. Pcmv, cytomegalovirus immediate–early promoter/enhancer; bGHpolyA, bovine growth hormone polyadenylation signal; ITR, AAV left and right inverted terminal repeat structures; hFIXm1, human factor IX cDNA containing truncated first intron.*

hemophilia B dogs (A72). The total dose of rAAV/hF.IX was 2.5×10^{12} virus particles in 0.5 ml, delivered through a 27 g needle in divided doses of 0.35 ml to the left hindlimb and 0.15 ml to the right hindlimb. The right hindlimb dose was admixed with carbon particles to aid subsequent localization of injection site. The animal was not treated prophylactically with canine F.IX and remained asymptomatic throughout the study period without evidence of bleeding at either injection site. The test animal did not display the generalized systemic reaction that has been described with repeated administration of exogenous human factor IX.[13,14] A panel of coagulation parameters was obtained before treatment and at least weekly throughout the study period (Figure 2). Baseline coagulation studies were characteristic of this strain of hemophilic animals, which produces no detectable canine factor IX.[13,16] Following administration of rAAV/hF.IX the whole blood clotting time (WBCT) was shortened on days 1 and 6, to values intermediate between the normal range (6–8 min) and the dog's hemophilic baseline (>60 min). Circulating human factor IX was not detected by our ELISA, with a limit of sensitivity of 1–2 ng/ml. Expression of the recombinant human factor IX was confirmed by the development of specific antihuman factor IX antibody by day 16 after infection (Figure 2). The presence of an antibody was suggested by the APTT mixing study and confirmed by the Bethesda inhibitor assay. Antibody response to hFIX was sustained and the absolute value (4–6 BIU) is the same as that reported for factor IX-deficient dogs treated with daily exogenous recombinant or plasma-derived human factor IX.[13] The appearance of antifactor IX antibody development following 2 weeks of exposure to protein expression is consistent with the response in hemophilic canines receiving plasma-derived hF.IX and recombinant hF.IX protein.[13,14]

Histologic analysis

At necropsy, performed 10 weeks after injection, no gross abnormalities were noted in any internal organs or within the injection sites. Hematoxylin and eosin (H & E) stained sections of internal organs and control muscle showed no viral cytopathic effects and no inflammation. Gross examination and histologic sections of the right semimembranosus muscle revealed carbon particles, primarily within loose connective tissue between muscle bundles and occasionally between individual myocytes (Figure 3a and d). Adjacent to the areas of heaviest carbon particle deposition there were scattered myocytes, usually single and occasionally in small clusters, which were surrounded by a rim of lymphocytes. Some of these myocytes also showed nonspecific degenerative changes such as internalized nuclei and hyalinization (Figures 3d and 4b). Areas more distant to the injection site (>3 mm) within the semimembranosus muscle showed no inflammation and no myocyte degeneration (Figures 3c and 4a).

Immunohistochemical evaluation with the AHIX-5041 antibody to human factor IX demonstrated marked expression of hF.IX in cross-sections of the transduced muscle (Figures 3b (right hindlimb muscle (RHL) portion) and 4c and f). The pattern of staining is homogeneous in some regions and more mosaiform in others. The intensity of cytoplasmic staining shows variability between cells, consistent with the pattern previously demonstrated in studies of murine muscle expression following rAAV reporter gene transduction.[8,12] Cross-sections of the transduced muscle tissue showed human factor IX present in myocytes up to 1.2 cm from the injection site (data not shown). Control muscle from the pectoral girdle was negative for cytoplasmic human factor IX (Figure 3b (pectoral muscle (P) portion)). Omission of primary antibody for human factor IX resulted in a loss of staining (data not shown) and provided further indirect support for the general conclusion of human factor IX expression only in the AAV-transduced muscle.

The pattern of lymphocytic infiltration was similar to previously described results using an adenoviral vector (Figures 3d and 4b).[11] Frequently, involved myocytes were negative for human factor IX (Figures 3f and 4d). Further investigation of the etiology of the lymphocytic infiltrate, using an antibody to adenovirus, demonstrated marked positivity in all areas of inflammation (Figure 4f). The adenovirus antigen was frequently nuclear in location, and was clearly present on internalized nuclei. Areas that were free of inflammation, yet strongly positive for human factor IX, were completely negative for adenoviral antigen (Figure 4e). These results support efficient spread of AAV

Sample Day	WBCT(min.)	PTT (sec.)	APTT Mixing Study (sec.)	B.I.U.
0	>60	91.4	28.7	
1	36	89.7	28.8	
3	>60	90.4	28.3	0
6	29	101.7	29.1	0
8	>60	[WBCT only]		
9	>60	88.9	29.8	
12	>60	82.1	41.5	
16	>60	89	59	4
20	>60	87.8	57.6	
23	>60	78.7	56.6	
27	>60	93.4	58.6	
30	>60	86.3	57.9	6
37	>60	88.1	56.9	
44	>60	[WBCT only]		

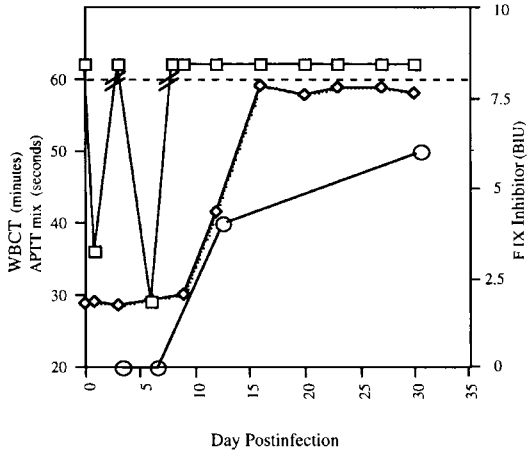

Figure 2 *Hemophilic dog hemostatic response and humoral immune response to expression of human factor IX. Left: Coagulation assays and anti-hFIX inhibitor antibody (expressed in Bethesda inhibitor units; BIU). Factor IX expression was not reflected in a change in PTT or ELISA. Right: Following WBCT (□) changes in the first week, all values returned to the hemophilic baseline of >60 min, represented by all values above dashed line. APTT mixing study (◇) prolongation and inhibitor antibody (○) titer parallel increases are shown.*

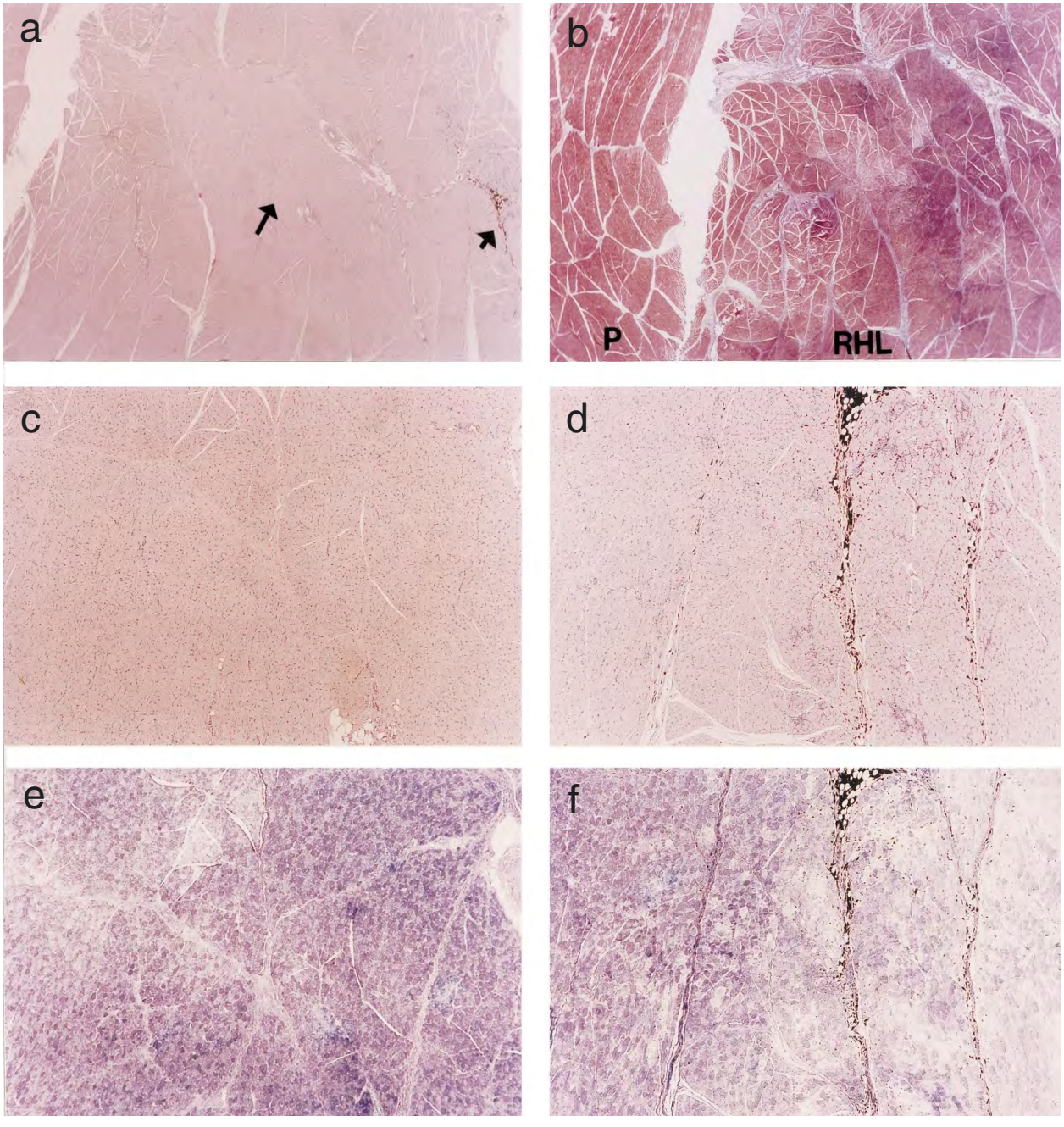

Figure 3 *Histologic analysis of transduced canine skeletal muscle at 10 weeks after injection of rAAV/hFIX. (a) Hematoxylin and eosin stain of entire cross-sectional area of medial semimembranous muscle (approximately 1.2 cm) right hindlimb. Carbon particles (adjacent to small arrow) are seen at injection site. (b) Immunohistochemical staining for hFIX with specific antibody/alkaline phosphatase (purple staining); counterstaining is with H & E. RHL, right hindlimb; P, pectoral muscle. (c) H & E stain of region adjacent to long arrow (a), demonstrating lack of muscle pathology. (d) H & E stain of region adjacent to short arrow in (a), demonstrating areas of lymphocytic infiltrate in proximity to injection site. (e) hFIX immunohistochemical stain in section adjacent to (c), distant from injection site. (f) hFIX immunohistochemical stain in section consecutive to (d), adjacent to injection site. Original magnification: (a, b) × 10; (c–f) × 40.*

vector (1.2 cm) in injected tissue with only localized immune response shown in tissues positive for adenovirus protein. Long-term (10 week) transgene expression was documented and, as in previous studies, no cellular immune response could be detected in the majority of muscle tissue efficiently transduced by rAAV.[3,12] Attempts to rescue live adenovirus from residual rAAV/hF.IX peak fraction were performed by infecting 293 cells with the residual virus. No adenovirus cytopathic effect could be observed over three passages in tissue culture, suggesting that live adenovirus remaining after heat kill steps was very low titer or absent. Presence of residual inactivated adenovirus proteins after heat kill was not investigated, but previous characterizations of rAAV preparations have confirmed that adenovirus structural proteins persist following cesium gradient and heat kill treatments (X Xiao, personal communication).

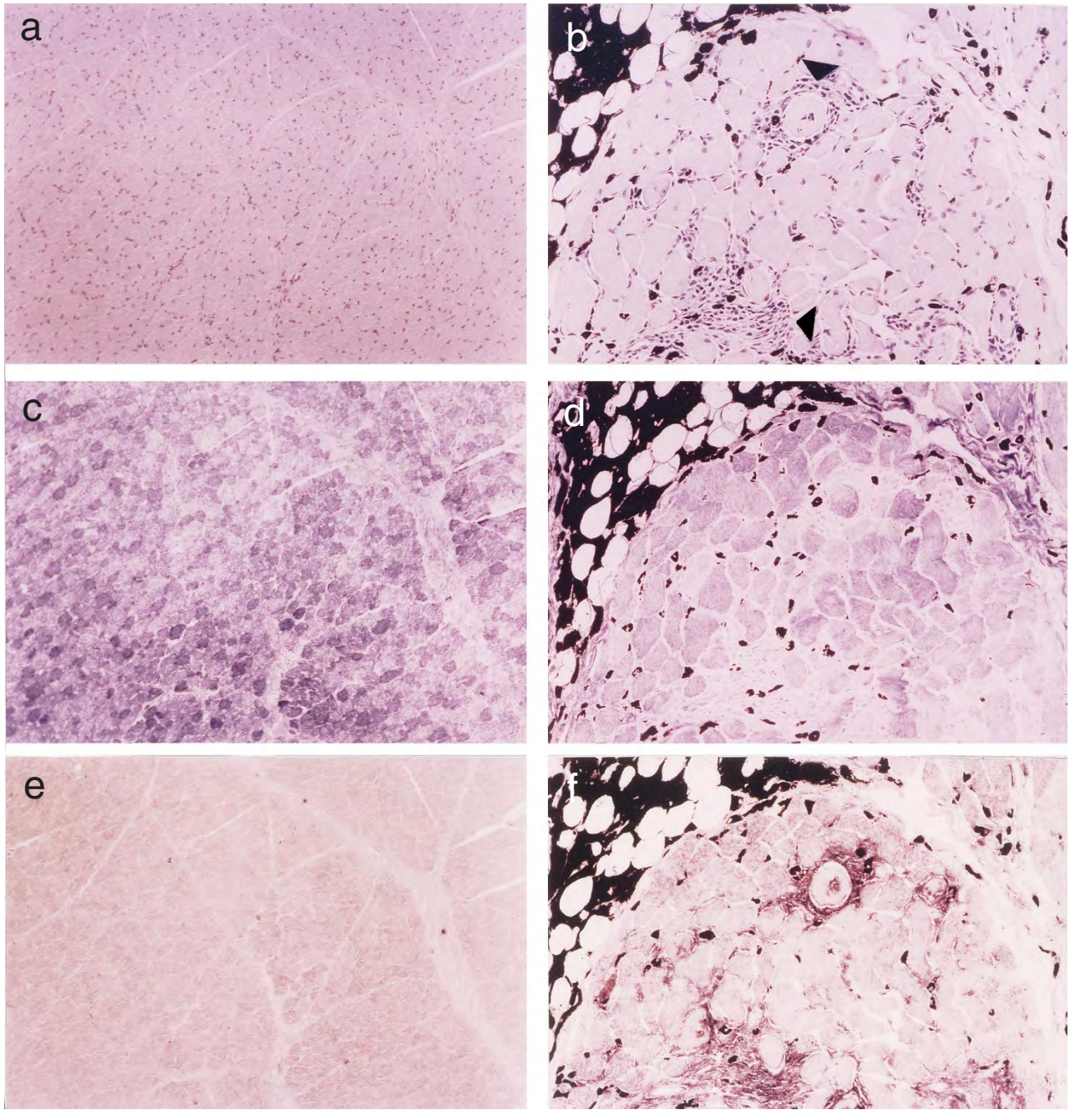

Figure 4 *Characterization of hFIX expression and local immune response in regions adjacent to (b, d, f) and distant from (a, c, e) injection site. (a, c, e) Region of long arrow in Figure 3a. (b, d, f) Region of short arrow in Figure 3a. (a) H & E stain. Uniform staining in myotube peripheral nuclei. (c) Anti-hFIX immunostain. Mosaic expression pattern in myofibers. (e) Anti-adenovirus immunostain, with H & E counterstain. (b) H & E stain. Large arrows indicate areas of focal lymphocytic infiltration and individual myocytes (some with central nuclei) rimmed by lymphocytes. (d) Anti-hFIX immunostain. Note uneven pattern of staining, showing strongest expression inversely correlated with infiltrate. (f) Anti-adenovirus immunostain. Note myocyte with central nucleus and both nuclear/peripheral staining. Original magnification: (a, c, e) × 100; (b, d, f) × 200.*

Molecular analysis of rAAV/hF.IX distribution and expression

No rAAV genomes were detected by PCR analysis of genomic DNA from pectoral girdle (control) muscle, brain, thymus, liver, lung, thyroid, adrenal gland, kidney, or testis (Figure 5). As expected, we detected the appropriate vector PCR product using DNA from the transduced right semimembranous muscle. DNA PCR of genomic DNA from the right inguinal lymph nodes was the only other positive signal detected (Figure 5). PCR of genomic DNA using primers specific for adenovirus generated the appropriate product using genomic DNA isolated from muscle at the injection site. Genomic DNA isolated from noninfected muscle yielded no adenovirus-specific PCR product, consistent with our immunologic observation.

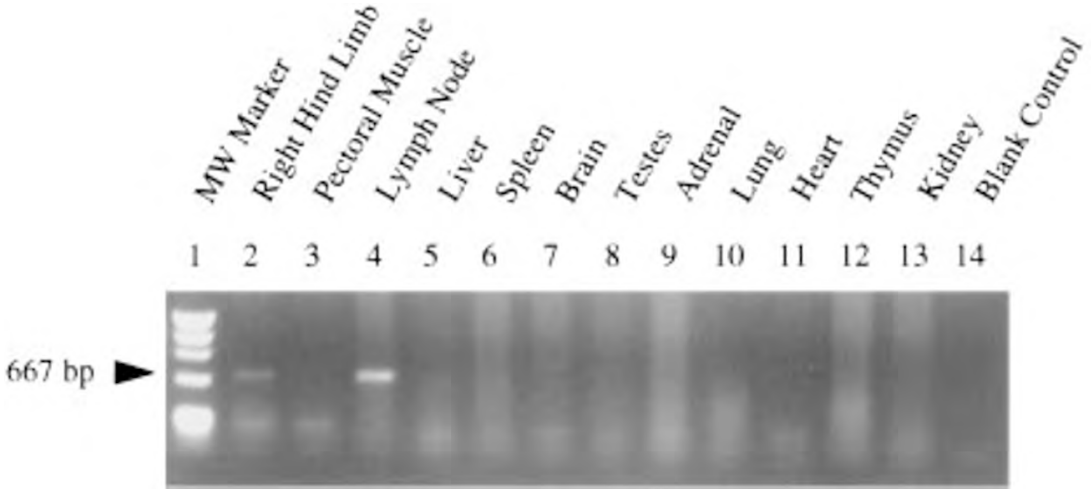

Figure 5 *DNA PCR amplification from genomic DNA of hemophilic dog tissues. Amplified 667 bp product includes sequences specific to pTRhFIXm1 first intron of human FIX cDNA (ethidium bromide staining). Lane 1: size marker: PhiXλ virus, BamHI digest; lane 2: target muscle from right hind limb; lane 3: control skeletal muscle from pectoral girdle; lane 4: lymph node from right inguinal region draining target area; lanes 5–14: panel of tissues from an injected dog, with no DNA control at far right.*

Discussion

This report describes the long-term expression *in vivo* of human factor IX using rAAV-transduced skeletal muscle in a hemophilia B canine. The expressed human factor IX was biologically active as measured by the sensitive whole blood clotting time (WBCT) assay. Expressed hF.IX antigenically mimicked human factor IX in a hemophilic canine resulting in the development of an antibody inhibitor. PCR analysis demonstrated the absence of vector spread to nontransduced tissue, including gonad and brain, with the exception of the lymph node draining the site of injection.

Experience with a wide variety of genes and gene delivery systems supports the notion that skeletal muscle can secrete transgenic protein products. Most muscle-directed approaches for factor IX expression have involved the *ex vivo* transduction of myoblasts, which can be re-implanted and result in secretion of factor IX into the circulation.[5,6,17] These studies have gone far to establish that both myoblasts and mature myotubes are capable of the synthesis and required post-translational modifications of factor IX protein. In addition to *ex vivo* approaches, secretion of circulating proteins following direct muscle injection has also been demonstrated. Circulating levels of factor IX following recombinant adenovirus injection, as well as human factor VII and human α-1-antitrypsin following plasmid DNA injection, have been achieved *in vivo* from skeletal muscle.[9,18,19] In the case of direct DNA injections, the secreted protein was detected in the mouse circulation at the end of the first week after injection, with subsequent disappearance of circulating protein due to a protein-directed antibody, similar to the pattern we observed.

The canine study was designed based on previous work in which we demonstrated efficient transduction and long-term expression of the reporter gene β-galactosidase in skeletal muscle of immunocompetent mice.[3] The dose of rAAV/hF.IX chosen for this experiment was extrapolated from the dose of rAAV/β-galactosidase injected intramuscularly (approximately 10^8 infectious units/kg). In this study, expression of the rAAV/hF.IX transgene *in vivo* resulted in a transient improvement of the bleeding phenotype, as reflected by decreases in the sensitive WBCT. This assay is sensitive to picogram quantities of F.IX, and expression in the first week after injection was presumably low, with WBCT shortening seen on days 1 and 6, but not day 3, after infection. Circulating hF.IX could not be detected at the limits of detection of our ELISA before the development of a detectable circulating antibody to the human protein during the second week after AAV delivery. This timing is consistent with the described pattern of antibody formation in dogs repeatedly challenged intravenously with human factor IX[13,14] and prevented us from determining final levels of secreted factor IX. For this reason, we cannot comment on the therapeutic efficacy of this dose. The persistence of the inhibitor antibody titer and corresponding elevated APTT mixing study, however, suggest sustained hF.IX expression in the animal. These observations were supported by immunohistochemical staining of rAAV-infected muscle for hF.IX expression at 10 weeks after injection (the duration of the experiment). Since other studies demonstrate successful recombinant human protein (erythropoietin) secretion from muscle after rAAV transduction,[8] we feel it is not likely that the dog's transduced skeletal muscle was unable to secrete the factor IX that we detected by immunohistochemistry. Instead, it is likely that the antifactor IX antibody rapidly complexed and cleared the secreted protein. This contrasts with the reported fate of erythropoietin secreted from rAAV-transduced murine skeletal muscle for up to 40 weeks. It is worth noting from that study that BALB/c mice tolerated recombinant human erythropoietin, while C57Bl/6 mice did not. In contrast, human F.IX specifically induces antibody formation in BALB/c mice, but has evaded antibody formation in C57Bl/6 mice.[20] The relative antigenicity of a recombinant protein in any given *in vivo* application appears to involve a complex interplay of the particular transgene, vector, animal strain and tissue targeted.[21,22] While these results support the safe transduction of the target muscle by rAAV vectors leading to human factor IX expression, we cannot rule out from our

studies that expressed factor IX may be bound locally, and thus sequestered from efficient delivery into the circulation.[23]

It is interesting to note that the level of expression of factor IX observed *in vitro* increased gradually over 1 week after infection, achieving levels comparable to those previously reported in hemophilic dog fibroblasts following retroviral or adenovirus-polylysine-DNA factor IX gene delivery.[4,24] This increase with time is not unexpected, as both *in vivo* and *in vitro* experiments suggest AAV transduction increases gradually, reaching a plateau in the first weeks after infection[3,8] (also, Snyder and Muzyczka personal communication). This gradual increase in rAAV gene expression may be related to a rate-limiting step in rAAV transduction that we and others have characterized *in vitro*.[25–28] The delay in expression from rAAV vectors presents an interesting parameter to consider in designing escalated dose studies in a clinical trial; evaluation of dose–response will require unique consideration of rAAV persistence and expression. While this study offers information regarding persistent expression and safety of the human F.IX AAV vector in a large animal model, further experiments using animals with compromised or absent antibody response will be required to determine the maximum expression at a given dose of rAAV/hF.IX. Alternatively, expression of the autologous protein (eg canine factor IX in the dog) could yield more precise data regarding the function of the recombinant protein. Safety data using the human gene, however, is a crucial preliminary in planning therapeutic human trials.

Transduction in the injected muscle was extensive and limited by the fascial border of the muscle. The immunohistochemical staining demonstrated that myotubes were transduced as far as 1.2 cm away from the injection site. Although AAV is among the smallest known viruses, it is not clear if the mechanism of spread was purely mechanical; co-injected carbon particles and contaminating adenovirus remain confined within approximately 2 mm of the injection tract. Mononucleated myoblasts spontaneously fuse with large multinucleated myotubes, 'sharing' their gene products.[29] Myoblasts are reportedly more efficiently transduced than myotubes by adenovirus recombinants;[30] the same comparison has not been done using rAAV. The efficient transduction of myotubes that we observed could result from primary AAV infection of differentiated myotubes or infection of myoblasts with subsequent formation of fusion myofibers. In our young animal, experiencing normal growth and development, a combination of mechanisms is possible.

An unexpected finding was scattered areas of lymphocytic infiltration in proximity to the injection tract. Cytotoxic lymphocyte (CTL) response to adenovirus vectors delivered to muscle is a predictable phenomenon, felt to be mediated by class I MHC-restricted $CD8^+$ and $CD4^+$ lymphocytes.[31,32] Cellular immune response following AAV vector delivery to muscle is absent[12] or limited to the period immediately following infection.[3] We have observed a striking histologic difference when recombinant adenovirus-β-galactosidase was compared with rAAV-β-galactosidase injection in mouse muscle. Cytotoxic and helper T cell activation were observed only in the Ad-infected regions.[3,12] We have also determined that in muscle infected with recombinant adenovirus-β-galactosidase alone or in areas co-infected with rAAV and rAd, cellular immune response resulted in removal of Ad-transduced cells within 3 weeks of infection, with preservation of AAV-transduced myocytes.[3] Areas of lymphocytic infiltrate in the study animal presented here consistently stained for adenovirus proteins, suggesting a low level of adenovirus or heat denatured adenovirus protein contamination in the rAAV preparation. The great majority of transduced cells, and particularly those regions with strongest rAAV transgene expression, showed no evidence of cellular immune response, consistent with the previous published results.[3,12] Residual staining for adenovirus at 10 weeks after infection suggests that canine cells were not as efficiently eliminated as might be expected based on the studies noted. Our *in vitro* studies with canine fibroblasts suggest that canine cells will infect with adenovirus (Ad dl309), though at a much lower level of permissiveness than human cells (normal host) and somewhat more slowly than cultured mouse myoblasts (data not shown). The semi-permissive nature of adenovirus infection in canine cells may allow limited continued presence of adenovirus protein in the absence of active infection. Additionally, it has recently been shown that incomplete or inactivated adenovirus particles can invoke similar degrees of cellular inflammatory response as infection-competent adenovirus.[33] Taken together, our data from hindlimb muscle PCR and immunostaining, along with our inability to rescue live adenovirus from the rAAV/hF.IX, support inactivated viral proteins as causing the infiltrate observed. Our study would suggest that further studies in which live helper virus or inactivated virus are completely removed from rAAV preparation will be required to ensure optimal safety in clinical trials.

In conclusion, this report of the intramuscular delivery of recombinant AAV-factor IX to a hemophilic dog highlights the promise of this vector. At the dose given, AAV is safe and results in local transduction of muscle tissue. More experiments are needed in the hemophilia B dogs to extend these findings but the data are consistent with the *in vivo* production of functional F.IX in sufficient quantities to shorten the WBCT and generate an antibody to human F.IX. *In vitro* analysis indicates protein production from the mammalian cells will be dose-dependent; this study suggests increasing the dose should be safe and yield incremental improvements in clinical response. The generation of antibody to human factor IX prevented quantification of transgenic protein expression, which is predicted to rise with time. This pattern of expression from rAAV vectors may make them unique among other factor IX delivery vectors previously used in muscle. AAV vectors remain difficult to produce in large quantities at high titers, despite ongoing efforts to reproduce packaging cell lines and other strategies employed for retroviral and adenoviral vectors. As this study showed, the current dependence on helper virus (adenovirus) functions for recombinant AAV production puts stocks at risk for contamination. Previously, we described the production of rAAV in the complete absence of infectious adenovirus.[25] Current efforts in our laboratory involve the optimization of production of recombinant AAV without the use of infectious helper virus, which will eliminate the possibility of adenovirus contamination and should accelerate the testing of this vector in humans. This report of the first use of AAV in a large animal model of human disease demonstrates the enormous potential that recombinant

AAV holds for intramuscular transgene delivery and underscores the importance of overcoming current production difficulties.

Materials and methods

Isolation of hemophilic dog fibroblasts and cell culture

Hemophilia B canine tissue was obtained from an adult male of the Chapel Hill strain. All animal surgical and care procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Tissues were minced and fibroblasts isolated using standard tissue culture methods. Fibroblasts, human embryonic kidney 293, and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were maintained in media containing serum depleted of bovine factor IX.[34] No factor IX was detected by ELISA from the treated serum or media, which were fully active in supporting cell growth.

Vector construction

The rAAV human factor IX expression vector was constructed using standard subcloning methods. Briefly, the human factor IX cDNA (hF.IX) including a truncated portion of the first intron was derived from p416FIXm1 kindly provided by Dr K Kurachi (University of Michigan).[15] Digestion with *Bam*HI yielded the hF.IXm1 minigene which contains the entire coding region of hF.IX cDNA and 981bp and 443 bp sequences from the 5′ and 3′-end regions of the factor IX first intron, respectively. The presence of intron I sequence has been reported to improve stability of F.IX mRNA.[15] The AAV vector plasmid pTR-UF2[35] was digested with *Bam*HI to yield a vector backbone with 5′ and 3′ AAV inverted terminal repeat structures, with the CMV immediate–early promoter/enhancer upstream of the *Bam*HI site, and the bovine growth hormone polyadenylation signal immediately downstream of the *Bam*HI site. The FIXm1 minigene was subcloned into the *Bam*HI site of the AAV vector backbone yielding pTRhFIXm1. This represents a final vector of 6909 bp with a 3.8 kb insert between the AAV ITRs (equivalent to 85% of wild-type AAV genome size).

Transient expression from plasmid

Factor IX expression derived from pTRhF9m1 was tested in a transient expression assay following calcium phosphate transfection of 293 cells. Expression of human factor IX into overlying media was confirmed by an ELISA using the Asserachrom IX:Ag ELISA kit (American Bioproduct, Parsipanny, NJ, USA), using the manufacturer's recommended protocol. A modified aPTT assay (ability of factor IX expressed by tissue culture cells to supplement F.IX-deficient plasma in coagulation intrinsic pathway, as assessed by fibrometer clotting time) was used to assess functional activity of hF.IX expressed into media.[36]

rAAV preparation

rAAV/hF.IX virus was produced as previously described.[3,37,38] Briefly, rAAV virions were generated by calcium-phosphate cotransfection of (7.5 μg) pTRhFIXm1 and (22.5 μg) helper plasmid ACG-2 and subsequently infected with adenovirus type 5 at a multiplicity of infection of 5 infectious units (IU) per cell. Cells were observed for characteristic adenovirus cytopathic effect (CPE) and harvested 48 h after infection. Cells were subjected to three successive freeze–thaw cycles and centrifuged to remove cell debris. rAAV virions were isolated by sequential high salt precipitation and cesium density gradient centrifugation. Fractions were further purified with a second CsCl gradient centrifugation (1.45 g/ml CsCl). The titer of rAAV was determined by a dot-blot hybridization assay. Following DNase digestion and proteinase K treatment, 5 μl samples from each fraction were denatured and probed with radiolabeled *Bam*HI fragment of the hF.IX cDNA plasmid. Serial dilutions of linearized plasmid pTRhFIXm1 were probed in parallel for DNA concentration standards, and signal intensity from gradient fractions evaluated to determine peak fractions and number of recombinant F.IX genomes per fraction, to yield a titer expressed as virus particles per ml. In our experience, the number of infectious units is on the order of 500–1000 times less than the rAAV particle number. rAAV was then dialyzed against 10 mm Tris, 140 mm NaCl, 1 mm MgCl, 3% glycerol with three dialysis changes over 4 h. Virus aliquots were subsequently heat-treated at 56°C for 15–20 min to inactivate wild-type adenovirus.

Laboratory stocks of rAAV/β-gal were prepared using the same methods as for rAAV/hF.IX, with the plasmid pAB-11 substituted for pTRhFIXm1, but were titrated using histochemical staining, as previously described,[3] rather than by dot-blot assay. In brief, 1 μl from each fraction of rAAV/β-gal was used to infect 293 cells in 24-well tissue culture plates, along with co-infecting adenovirus dl309 at an MOI of 2. Cells were fixed at 24 h post-infection, stained with 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal), and examined for nuclear blue staining. Each blue cell was accounted as transduced by one infectious rAAV/β-gal particle, to yield a titer expressed in infectious units (IU)/ml.

rAAV/hF.IX expression in cultured cells

Primary hemophilic dog fibroblasts were plated into six-well tissue culture plates (Corning; Costar, Cambridge, MA, USA) at a density of 1×10^5 cells per well in DMEM-H media containing serum depleted of all vitamin K-dependent factors, including bovine factor IX, using barium sulfate precipitation and supplemented with vitamin K (500 ng/ml). Twelve hours later, cells were infected at the following concentrations of rAAV/hF.IX (virus particles per cell): 0 parts per cell, 1×10^3 parts per cell, 1×10^4 parts per cell, 5×10^4 parts per cell, 1×10^5 parts per cell, and 5×10^5 parts per cell. Viral infection lasted 2 h and then media were replaced with a total of 2 ml. After 24 h, and every 24 h subsequently for 2 weeks, the media were collected, frozen at −80°C, and replaced with 2 ml of fresh factor IX-depleted media. When primary fibroblasts became confluent at day 7, cells were trypsinized, harvested, counted, and replated at the original cell density (1×10^5 cells per well). Twenty-four hour collections were then resumed after allowing 12 h for cells to adhere after passage.

Quantification by ELISA

Human factor IX in samples was quantified by ELISA. Ninety-six-well EIA plates (Costar) were coated with the mouse monoclonal anti-human factor IX antibody AHIX-5041 (Haematologic Technologies, Essex Junction, VT,

USA) in 0.1 m sodium carbonate buffer at 4°C overnight. Plates were blocked for 1 h with PBS/0.05% Tween 20/0.25% BSA at room temperature. Samples of 0.1 ml of medium overlay or plasma were applied and incubated at room temperature for 2 h. Peroxidase-conjugated goat antifactor IX polyclonal antibody (Enzyme Research Laboratories, South Bend, IN, USA) was added as the detecting antibody and incubated for 2 h at room temperature. ABTS ELISA substrate solution (Boehringer Mannheim, Indianapolis, IN, USA) was added to effect color development for 30 min and absorbance measured at 405 nm with a Bio-Tek EL311 microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Factor IX levels were calculated by comparison with a standard curve generated from serial dilutions of Factor IX assayed from normal human plasma (George King Bio-Medical, Overland Park, KS, USA). Factor IX values as low as 1–2 ng/ml were reproducibly measured.

Screening of Hemophilia B canines for antibodies to rAAV

Hemophilia B canines (Chapel Hill strain), which are routinely immunized to canine parvovirus, were screened for antibodies against AAV/parvovirus. Serum samples from six animals were tested for their ability to interfere with AAV infection of HeLa cells. HeLa cells were plated at 2×10^5 cells per well plate and infected with rAAV carrying the marker gene β-galactosidase at a multiplicity of infection of either 0.1 or 1.0 infectious units per cell. HeLa cells were also infected with adenovirus dl309 at an MOI of 5 (1×10^6 IU per well) for 1 h to provide helper function for optimal rAAV/β-gal expression. rAAV/β-gal was preincubated with varying amounts (0, 1, 10 and 100 μl) of dog serum brought to a total volume of 500 μl using serum-free DMEM-H tissue culture media. The serum-rAAV mixture was incubated at 37°C for 1 h with continuous rocking. As a control, 0.1 μl of guinea pig polyclonal anti-AAV antibody was incubated with rAAV under the same conditions and proved to inhibit AAV transduction of HeLa cells completely**.** Following serum incubation, HeLa cells were infected for 1 h at 37°C with frequent rocking. Cells were fixed at 24 h after infection and stained for β-galactosidase activity with 5-bromo-4-chloro-3-chloro-indolyl-β-d-galactopyranoside (X-gal).

In vivo viral delivery and transgene expression

The hemophilia B canines were maintained at the Francis Owen Blood Laboratory of the University of North Carolina at Chapel Hill in accordance with the guidelines of the UNC Institutional Animal Care and Use Committee. The animal (A72), screened negative for antibodies capable of inhibiting rAAV infection, was treated with rAAV/hF.IX at 9 weeks of age (4.5 kg). A total dose of 2.5×10^{12} viral particles in 0.5 ml (the single peak fraction of one virus preparation) was administered intramuscularly in divided doses to the left (0.35 ml) and right (0.15 ml) hindlimb semimembranosus muscles, with the dose to the right limb admixed with carbon particles to aid in histologic localization. Blood was collected on the sample days indicated and coagulation assays included the whole blood clotting time (WBCT), PTT, determination of Bethesda anti-factor IX inhibitor units (BIU) as previously described,[13,39] as well as the factor IX ELISA described above. The standard APTT mixing study was performed as a screen for human F.IX inhibitor development.

At 10 weeks after virus administration, the animal was killed with intravenous pentobarbital overdose and tissues were collected for histologic evaluation. Portions of right hindlimb musculature, including the area marked by carbon particles, pectoral girdle musculature, and all major organs were either rapidly frozen or fixed in 10% neutral-buffered formalin overnight. Frozen tissue was homogenized and genomic DNA and total RNA extracted using standard techniques. Formalin fixed tissue was routinely processed and paraffin-embedded. Tissue was sectioned at 6 μm, deparaffinized in xylene, rehydrated through graded ethanols, and either stained with hematoxylin and eosin (H & E) or immunohistochemistry performed. Mouse monoclonal antihuman factor IX antibody AHIX-5041 (Haematologic Technologies) was applied at 2 μg/ml of phosphate-buffered saline (pH 7.5) with 10 mm NaPhosphate, incubated at 37°C for 4 h, followed by incubation with goat-derived antimouse IgG conjugated to alkaline phosphatase (Boehringer Mannheim), color development used NBT/BCIP (Boehringer Mannheim), for 1 h. Slides were counterstained with eosin, and dehydrated. Consecutive sections were treated with 0.25 μg proteinase K for 10 min at room temperature, then incubated with 1 μl/ml buffer of rabbit anti-adenovirus type 5 antibody (NIAID), followed by incubation with goat anti-rabbit antibody conjugated to alkaline phosphatase (Boehringer Mannheim).

Genomic DNA isolation and DNA PCR of animal tissue

Animal organs including gonads, brain, liver, spleen, lymph nodes, hindlimb skeletal muscle, pectoral girdle muscle and lung were harvested and snap frozen at –80°C. Samples were then processed for genomic DNA isolation using proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. DNA PCR analysis employed primers unique to the pTRhFIXm1. The upstream primer used AGACATGATAGCTGGACC located in the hF.IX intron and the downstream primer ATTGGACTCACACTGATCTCC located in exon 6. The PCR reaction conditions used those recommended by the manufacturer (Perkin Elmer-Cetus, Norwalk, CT, USA) which included 1.0 μg of genomic DNA. Amplification conditions were 94°C for 3 min (one cycle), 94°C for 45 s, 65°C for 1 min, and 72°C for 45 s (10 cycles), 94°C for 30 s, 62°C for 45 s, 62°C for 45 s (17 cycles), 94°C for 30 s, 60° for 30 s, 72°C for 30 s (eight cycles). Samples were electrophoresed on a 0.8% agarose gel containing ethidium bromide. Primers for amplification of adenovirus genomic DNA kindly provided by UNC Cystic Fibrosis Center. PCR of adenovirus DNA used the same reaction profile and reaction mixture concentrations noted above.

Efforts to rescue contaminating adenovirus from rAAV/hF.IX

Residual virus remaining in the injection apparatus was stored in Tris-EDTA pH 8.0 at –80°C. To examine for live adenovirus contamination, this residual virus was used to overlay 293 cells plated at 3×10^5 cells per well in a 12-well tissue culture plate. 293 Cells were followed for three passages and observed for typical morphology of adenovirus cytopathic effect (CPE). Additionally, 293 cells grown to confluence in the presence of residual virus were subjected to three serial freeze–thaw cycles and this cell lysate used to overlay fresh 293 cells, which

were subsequently followed for evidence of adenovirus lytic cycle CPE.

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