Regional intravascular delivery of AAV-2-F.IX to skeletal muscle achieves long-term correction of hemophilia B in a large animal model

Valder R. Arruda¹*, Hansell H. Stedman²*, Timothy C. Nichols³, Mark E. Haskins⁴, Matthew Nicholson⁴, Roland W. Herzog¹, Linda B. Couto⁵, Katherine A. High¹⁶§

¹Hematology Division, Department of Pediatrics, Children’s Hospital of Philadelphia, ²Department of Surgery, University of Pennsylvania School of Medicine, ³Francis Owen Blood Research Laboratory, Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, ⁴School of Veterinary Medicine, University of Pennsylvania, ⁵Avigen, Inc., Alameda, CA, ⁶Howard Hughes Medical Institute, Children’s Hospital of Philadelphia

*These two authors contributed equally to the manuscript

Corresponding Author:
Katherine A. High, M.D.
The Children’s Hospital of Philadelphia
3615 Civic Center Boulevard
302 Abramson Research Center
Philadelphia, PA 19104
Tel: (215) 590-4521
Fax: (215) 590-3660
Email: high@email.chop.edu
ABSTRACT

In earlier work, we showed that adeno-associated virus mediated delivery of a Factor IX gene to skeletal muscle by direct intramuscular injection resulted in therapeutic levels of circulating Factor IX in mice. However, achievement of target doses in humans proved impractical because of the large number of injections required. We used a novel intravascular delivery technique to achieve successful transduction of extensive areas of skeletal muscle in a large animal with hemophilia. We provide here the first report of long-term (>3 years, with observation ongoing), robust Factor IX expression (circulating levels of 4-14%) by muscle-directed gene transfer in a large animal, resulting in essentially complete correction of the bleeding disorder in hemophilic dogs. The results of this translational study establish an experimental basis for clinical studies of this delivery method in humans with hemophilia B. These findings also have immediate relevance for gene transfer in patients with muscular dystrophy.
INTRODUCTION

In previous work, we showed that intramuscular injection of a recombinant AAV-2 vector expressing blood coagulation Factor IX (F.IX) resulted in long-term expression of F.IX, as judged by circulating F.IX levels and by immunohistochemistry of biopsied injected muscle, in mice and hemophilic dogs\(^1,2\). We also showed that safety and efficacy considerations imposed an upper limit on the amount of vector that could be injected into a single site; skeletal muscle has only a limited capacity to execute essential post-translational modifications\(^3\), the likelihood of forming neutralizing antibodies to F.IX, an undesirable consequence, rises as the AAV dose per site is increased\(^4\), and vector uptake into target cells is receptor-mediated and saturable\(^5\). This work formed the basis for a clinical trial in which an AAV-F.IX vector was administered by direct intramuscular injection into skeletal muscle of adults with severe hemophilia B\(^6,7\). The study had a dose escalation design; based on pre-clinical studies, the dose per site in the clinical trial was held to \(<2 \times 10^{12}\) vector genomes (vg) per injection site. The first subjects enrolled received only about ten injections, but the limitation on dose per site meant that subjects in the third and highest dose cohort required close to one hundred intramuscular injections in order to receive a dose of \(2 \times 10^{12}\) vg/kg. Based on pre-clinical studies in mice and dogs, the dose required for efficacy was \(~10^{13}\) vg/kg, but the number of injections required for this (\(~500\) sites) seemed prohibitive from a feasibility standpoint, and the target dose was not reached. Instead, the study was stopped after finding subtherapeutic levels of F.IX (an outcome consistent with dose-finding studies in the dog model) in two human subjects injected at a dose of \(2 \times 10^{12}\) vg/kg. Three important conclusions from the human study were that: 1) intramuscular injection of
AAV-F.IX at doses up to $2 \times 10^{12}$ vg/kg in humans was safe, with no evidence of toxicity; 2) the characteristics of skeletal muscle transduction by AAV-2 were similar in mice, dogs, and humans; and 3) transgene expression appeared stable over time, as judged by immunofluorescent staining of muscle biopsies examined up to 10 months after vector injection. We also concluded that administration of the target dose of vector would require a technique that allowed transduction of large numbers of muscle fibers without doing hundreds of intramuscular injections. In this report, we describe and validate such a technique in a large animal model of hemophilia B.
METHODS

AAV vector construction and production

Recombinant AAV-2 vectors were produced by triple transfection as previously described, using plasmids expressing canine F.IX (cF.IX) or LacZ under the control of the CMV promoter/enhancer, a plasmid supplying adenovirus helper functions, and a third plasmid containing the AAV-2 rep and cap genes.

Animal experiments

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania, or at the University of North Carolina at Chapel Hill. Sedation was achieved with sodium pentothal (11 to 29 mg/kg) and anesthesia was maintained with 1-4% isoflurane.

Intravascular delivery of rAAV to the skeletal muscle of hemophilia B dogs.

Four hemophilia B dogs underwent femoral artery and vein isolation. Two overlapping tourniquets were placed transmuscularly at the level of the proximal thigh, followed by systemic heparinization at a dose of 70 U/kg injected intravenously. Heparin effect was confirmed by prolongation of activated clotting time. After tourniquets were tightened, microvascular clamps were placed to occlude the femoral vessels. The hindlimb circulation was then perfused by arterial infusion of phosphate-buffered saline (PBS). Intra-arterial bolus injection of 1mg/kg body weight of papaverine was followed by 2.5 ml/kg of 10 mM histamine/PBS at pH 7.4. Five minutes later, AAV-cF.IX vector was infused at doses ranging from 1.7x 10^{12} vg/kg to 3.9 x10^{12} vg/kg in a volume of 2.5 ml/kg.
of a solution of 10 mM histamine/PBS, at pH 7.4 followed by a 'chase' with 10ml/kg of PBS. After 15-20 minutes of the perfusion/infusion procedure, the limb circulation was flushed with 15 ml/kg of PBS. Before release of the tourniquets, animals received IV infusion of cimetidine 10 mg/kg and benedryl 2.5 mg/kg. The vessels were sutured as the cannulae were withdrawn, the clamps on the femoral vessels and tourniquets were removed, and the incision was closed with resorbable suture. Reconnection of limb vessels to systemic vasculature was routinely associated with a brief period of hypotension (1-10 min) that responded readily to volume expansion with normal saline. Three dogs received cyclophosphamide at doses of 200-250 mg/m² of body surface one day prior to the day of the surgery and weekly thereafter up to 6 doses. One control animal (E60) did not receive cyclophosphamide. Pooled normal canine plasma was infused prior to the surgical procedure at doses calculated to achieve at least 30% normal cF.IX plasma levels. Muscle biopsies of the tibialis anterior of the perfused limb were taken in two dogs 2 to 3 years following vector administration.

Peripheral vein delivery of rAAV

To determine whether intravenous vector infusion by peripheral vein resulted in transgene expression, we injected a fifth hemophilia B dog at a dose of 2.9 x10¹² vg/kg rAAV-cF.IX and cyclophosphamide as described above.

Regional intravascular delivery of rAAV to the skeletal muscle of normal dogs

Two hemostatically normal dogs underwent an identical surgical procedure prior to experiments in hemophilia B dogs. One dog (6.5 kg) received infusion of rAAV-LacZ at
a dose of $3 \times 10^{12} \text{vg/kg}$; four weeks later muscle was biopsed. A second animal (11 kg) received rAAV-F.IX at a similar dose ($3 \times 10^{12} \text{ vg/kg}$) by limb perfusion. In the contralateral limb, vector from the same lot was introduced by percutaneous injection at one site of the tibialis anterior at a dose of $2 \times 10^{12} \text{ vg}$. Eight weeks post-injection the animals were sacrificed and biopsied at the IM injected site. In the perfused limb 18 random muscle biopsies were taken.

**Clotting assays and cF.IX antigen**

cF.IX concentration was determined by ELISA using as capture antibody at a dilution 1:800 sheep anti-cF.IX (Affinity Biologicals, Hamilton, ON, CA). A rabbit anti-cF.IX antibody (Affinity Biologicals, Hamilton, ON, CA) dilution of 1:1000 was used as secondary antibody, and for detection a 1:2000 swine anti-rabbit IgG peroxidase-labeled (Dako, Carpinteria, CA). F.IX clotting activity was determined by one-stage aPTT. Plasma test samples were mixed with canine F.IX deficient plasma and the aPTT values were compared with a reference standard consisting of serial dilutions of normal canine plasma mixed with F.IX deficient plasma. Bethesda assays were carried out for detection of inhibitory antibodies to cF.IX as previously reported.

**Histology and Immunohistochemistry**

Muscle sections were stained with hematoxylin and eosin for histology. Muscle serial cryosections (5-10 $\mu$m) were stained for cF.IX expression by immunofluorescence using a 1:100 dilution of rabbit anti-cF.IX antibody (Affinity Biologicals, Hamilton, ON, CA), and as secondary antibody a fluorescein-conjugated swine anti-rabbit IgG diluted at 1:50
(Dako, Carpenteira, CA). For double staining, rat anti-heparan sulfate proteoglycan (HSPG; 1:100, Chemicon, Temecula, CA) was applied simultaneously with rabbit anti-cF.IX at 1:100. The detection of HSPG staining was by a fluorescein-conjugated murine anti-rat IgG (Sigma, St Louis, MO) and F.IX staining was detected by incubating with rhodamine-conjugated goat anti-rabbit IgG (Sigma, St Louis, MO).

**Biodistribution and toxicity**

Serial blood cell counts and biochemical analysis of serum samples for liver and kidney function tests, and muscle enzymes were performed as described before ¹. We used a polymerase chain reaction (PCR)⁹ to detect vector sequences in serum of all dogs and tissues from normal dogs (skeletal muscle of hindlimbs, liver, kidney, lung, heart, and gonads). The sensitivity of detection was 30 vector genomes per μg of DNA.
RESULTS

Regional intravascular delivery of rAAV to the skeletal muscle results in widespread transgene expression in a large animal

We sought to determine whether a viral vector delivery technique that had been described in hamsters and rats \(^{10}\) could show efficacy in a large animal model. The technique involves vascular delivery of vector to the muscle tissue in a single limb. Briefly, the common femoral vessels are exposed, the animal is heparinized, the vessels are cannulated, and a tourniquet is securely tightened at the level of the hip joint. The now-isolated limb vasculature is perfused with a solution containing papaverine to effect vasodilatation. Five minutes later the limb circulation is infused with vector mixed with histamine, to induce vascular leakage. After perfusion/infusion, the limb circulation is thoroughly flushed and the tourniquets and clamps removed. Greelish et al. showed that this technique allowed extensive transduction of skeletal muscle with either adenovirus or AAV vectors in rats and hamsters \(^{10}\). In the current study, initial experiments were carried out in normal dogs to determine whether the procedure has relevance for gene transfer in larger animals. The first animal (6.5 kg) was infused with AAV-lacZ at a dose of \(3 \times 10^{12} \text{ vg/kg}\), with heparin, papaverine, and histamine doses scaled up linearly from the rodent studies on a per kg basis. A muscle biopsy taken 4 weeks later was uninformative in terms of gene transfer, showing only a cellular infiltrate and destruction of muscle architecture (data not shown). Based on earlier studies with AAV-lacZ in dogs (VRA and KAH, unpublished observations), we suspected that this was due to an immune response to \(\beta\)-galactosidase in dogs, and we repeated the experiment using
cF.IX, a protein to which normal dogs are fully tolerant. This second dog was injected by isolated limb perfusion in one hindlimb with AAV-CMV-cF.IX at a dose of $3 \times 10^{12}$ vg/kg, and also received a dose of $2 \times 10^{12}$ vg of the same vector at a single site, by direct IM injection in the opposite limb. Eight weeks later, the animal was sacrificed and muscle tissue harvested for immunofluorescence staining with an antibody to canine F.IX. In the hindlimb that received direct intramuscular injection, cF.IX expression was confined to the sites of injection (Figure 1a, b), with a radius of diffusion of ~0.5 mm, whereas the hindlimb that received vector by the intravascular infusion process (Figure 2) showed transduction throughout the muscle groups supplied by the injected vessel, with more extensive transduction around the stifle joint (corresponds to knee joint in humans) than at distal sites. Systemic levels of transgene expression could not be quantified in normal dogs, since the endogenous protein and the donated gene product are identical. A biodistribution study was performed on this animal at the time of sacrifice (8 weeks after vector infusion), using a PCR assay that can detect as few as 30 copies in 1 µg DNA. Of all tissues sampled, only perfused skeletal muscle was positive for vector sequences. Liver, lung, kidney, spleen, ovary, and contralateral muscle were all negative. The same assay showed that serum was positive for vector sequences for 5 days after vector infusion but was uniformly negative thereafter (data not shown).

**Long-term correction of hemophilia B phenotype by regional intravascular delivery of rAAV to the skeletal muscle of hemophilia B dogs**

To achieve a more quantitative analysis, and to determine safety and efficacy in an animal model of the human disease hemophilia B, we carried out the same vector
delivery procedure in hemophilia B dogs. These animals have disease due to a missense mutation in the portion of the gene encoding the catalytic domain of the protein. The animals have no detectable F.IX antigen or activity, so transgene expression can be assessed by both clotting assays, and by ELISA for expressed protein. Performance of the procedure in hemophilic animals required two modifications. First, animals were infused prior to and after the procedure with normal canine plasma, to insure adequate hemostasis for the surgical maneuver and for post-operative healing, and second, animals were immunosuppressed transiently with cyclophosphamide, for a period of 6 weeks, because we had shown in earlier studies that this maneuver reduced the risk of formation of inhibitory antibodies to F.IX when vector expressing cF.IX was administered to skeletal muscle in hemophilic animals. Since cyclophosphamide can lead to hemorrhagic cystitis, a complication that can be difficult to control in hemophilic animals, the dogs were also treated with MESNA (sodium 2-mercaptoethane sulfonate), a bladder cytoprotective agent. The first animal (D99) was infused at a dose of $3.7 \times 10^{12}$ vg/kg (Figure 3a). This resulted in circulating levels of cF.IX in the range of 600-800 ng/ml by ELISA, and 15% of normal human plasma levels by activity assay (Table 1) determined in several time points (ranging from 2 to 26 months) following ILP. This level of expression was sufficient to correct the activated partial thromboplastin time (aPTT) to a near normal value (Table 1, Figure 3b). These levels have been sustained for approximately 3 years, with observation ongoing. There was no drop in F.IX level and no appearance of neutralizing or non-neutralizing antibodies to F.IX after cyclophosphamide infusions were stopped at 6 weeks post vector infusion. Since prophylaxis against spontaneous bleeding episodes does not require levels of 15%, we
infused AAV-CMV-cF.IX at a lower dose ($1.7 \times 10^{12}$ vg/kg), again accompanied by short-term administration of cyclophosphamide and MESNA, and observed long-term expression of cF.IX in the range of 260 ng/ml by ELISA and 5.2% by activity. This was also accompanied by a shortening of the aPTT (Table 1, Figure 3b). A third experiment resulted in circulating levels of 210 ng/ml (4.2% by activity, with shortening of the aPTT), after a dose of $3.0 \times 10^{12}$ vg/kg. Whether the lower F.IX level seen in the third dog results from biological differences in the canine subjects, or minor variations in surgical technique, is unknown. However, it should be noted that all three experimental animals achieved long-term expression of canine F.IX at therapeutic levels. The excellent correlation between antigen and activity levels suggests that, at this level of expression, F.IX synthesized in skeletal muscle is fully biologically active. As further proof of this, it should be noted that, in the 74 months of cumulative observation of the three treated dogs, there have been only two bleeding episodes requiring treatment. The expected number based on observation of the colony over many years is $\sim 5.5$ episodes/12 months, or 34 over this period of observation \(^{14}\). As a comparison for efficacy, we have shown a historical control in which a dog from the same colony was injected with the same vector (AAV-CMV-cF.IX) at a comparable dose ($3.4 \times 10^{12}$ vg/kg) by direct intramuscular injection, with resulting circulating F.IX levels of $<1\%$ (Figure 3). Thus vector delivery by isolated limb perfusion results in higher circulating F.IX levels at comparable doses of vector.
**Inhibitor formation to cF.IX occurs in the absence of transient immunosuppression following regional intravascular delivery to skeletal muscle**

As a second control, isolated limb perfusion was repeated at a dose of \(3.9 \times 10^{12}\) vg/kg, but this time omitting cyclophosphamide and MESNA. In this instance, the animal formed a neutralizing antibody first detected \(~21\) days after vector administration, and persisting approximately \(300\) days (Figure 4). It should be noted that, even though the ELISA detects a low level of circulating cF.IX (Figure 4a), there is no correction of clotting times, with the aPTT remaining \(~50\) sec (Figure 4b) and F.IX clotting activity \(<1\)% (Table 1). This discrepancy between antigen and activity levels is typically seen in the presence of a neutralizing antibody (clinically termed an inhibitor) (Figure 4c). The development of the inhibitor under these circumstances suggests that the transient immunosuppression cannot be omitted.

**Peripheral intravenous delivery of rAAV to a hemophilia B dog results in subtherapeutic F.IX levels**

In a control experiment, a hemophilic animal was infused by peripheral vein with AAV-CMV-cF.IX at a dose of \(2.9 \times 10^{12}\) vg/kg, accompanied by short-term administration of cyclophosphamide and MESNA. The resulting level of F.IX was \(~50\) ng/ml, or approximately \(1\)% of normal circulating levels (Figure 3a), with a corresponding absence of positive fibers on muscle biopsy (\textit{vide infra}, Figure 5g). Thus it seems unlikely that the procedure will be successful as a simple IV infusion, at least with this AAV serotype.
**Route of vector delivery to skeletal muscle does not alter patterns of rAVV-2 transduction**

Muscle biopsies of the perfused limb (taken from the tibialis anterior) documented that transduction had occurred in a mosaic pattern (Figures 5a, d) similar to that seen with direct intramuscular injection in mice, dogs and humans \(^1,2,7\). Immunofluorescent staining of sections for heparan sulfate proteoglycan (HSPG), which acts as a receptor for AAV-2 \(^5\), indicates that fibers rich in HSPG are preferentially transduced by AAV-2 (Figures 5b, e), an observation confirmed on the colocalization experiment (Figures 5c, f). As shown previously in mice by Huard and colleagues \(^15\), and in humans by our group \(^7\), HSPG is abundant in slow fibers but not in fast fibers, accounting for the checkerboard pattern of positivity in transduced muscle. These results indicate that this property of AAV-2 transduction is not altered by the regional intravascular delivery method.

Hematoxylin and eosin staining of muscle biopsied 2 years after vector administration showed no evidence of muscle injury or inflammation, and continued expressin of cF.IX (Figure 5h)

**Biodistribution and toxicity**

The procedure was generally well-tolerated in both normal and hemophilic dogs, although a transient drop in blood pressure typically occurred on re-connection of the limb vasculature to the systemic circulation, presumably due to diffusion of residual histamine into the systemic vasculature (see Methods). Clinical laboratory studies including electrolytes, BUN, creatinine, liver function tests, and CBC were all normal in the days and weeks post-procedure. Creatine phosphokinase typically spiked to 3-7 fold
upper limits of normal immediately after the procedure, with a return to normal within the first week. Transient elevation of CPK is routinely observed in humans undergoing isolated limb perfusion\textsuperscript{16}. Results of biodistribution studies were similar to those obtained in normal dogs, with serum transiently positive for vector sequences up to day 5 after vector infusion (data not shown).
DISCUSSION

The development of efficient methods for bringing about targeted disruption of genes in murine embryonic stem cells opened a new line of investigation in biomedical research through the generation and characterization of murine models of human disease. Efforts quickly progressed from studies of pathophysiology to generation and testing of novel therapeutic strategies. In the realm of therapeutics, as in pathophysiology, murine models have proven deceptive as well as informative. Their small size guarantees that even difficult-to-produce reagents can be made in sufficient quantities, and it also facilitates delivery of test reagents in high doses. Other aspects of physiology critical for drug delivery, therapeutic feasibility, or toxicity may be only poorly modeled in mice, and, in the case of gene therapy, inbred strains of mice can be uninformative in terms of immune response to the vector or the transgene product. Thus one can enumerate at least ten novel therapeutic strategies that have cured hemophilia in mice, but none have yet dose so in humans. The myriad differences in small and large animal physiology underscore the fundamental importance of translational studies of novel therapies in large animal models as a necessary screen before contemplating human studies.

In our initial attempts to develop a clinical protocol for gene transfer in hemophilia B, we used skeletal muscle as the target tissue for an AAV vector expressing blood coagulation Factor IX. Compared to liver, the natural site of synthesis of F.IX, muscle is a desirable target tissue because it can be accessed in a relatively non-invasive fashion (IM
injection), that does not give rise to widespread biodistribution of vector$^{9,33,34}$. Moreover, the high prevalence of hepatitis B/C in the adult hemophilia population$^{35,36}$ (due to infection from older plasma-derived products) makes use of liver as the target tissue problematic. Therefore, we chose skeletal muscle as the target tissue in the first clinical studies of parenterally administered AAV vector. These studies established that intramuscular administration of AAV-CMV-F.IX was safe at doses up to $2 \times 10^{12}$ vg/kg in men with severe hemophilia B, and that there was long-term persistence of F.IX expression in injected muscle as judged by immunofluorescent staining of the tissue$^7$. This latter is a particularly important point, since long-term expression may be more difficult to achieve in AAV-2-mediated vector delivery to hepatocytes in humans$^{37}$. However, the clinical study and concurrent large animal studies documented limitations to the muscle delivery method that had not been anticipated from the initial studies in hemophilic dogs. First, it became clear in animal models of hemophilia that the risk of forming inhibitory antibodies to F.IX was higher after intramuscular delivery of AAV vector than after delivery of vector to the liver$^{1,4,12,13,38}$. Critical determinants included the dose of vector delivered per site, and the underlying mutation in the F.IX gene, which affects the animal’s immunological tolerance to the F.IX protein. To minimize the risk of inhibitor formation in the clinical study, the dose per site was kept below a limit that had been defined in the canine hemophilia model$^4$, and enrollment in the trial was limited to those with disease due to a missense mutation, a subgroup similar to the canine model, and at lower overall risk of inhibitor formation based on studies with protein infusion therapy$^{39,40}$. Other limiting factors included the low efficiency of skeletal muscle at executing certain critical post-translational modifications of the F.IX protein$^3$. 
and the fact that vector uptake is receptor-mediated, which sets an upper limit to the amount of vector that can gain access to cells at a single injection site. Using the experimentally determined constraints on vector dose per site ($2 \times 10^{12}$ vg/site), the procedure required an excessive number of injections merely to achieve a dose of $2 \times 10^{12}$ vg/kg, still substantially short of the target dose of $1 \times 10^{13}$ vg/kg. Without a more efficient delivery method, further dose escalation seemed impractical.

A more subtle point about vector delivery was also at play in the scale-up from mouse to dog to human. A dose of $1 \times 10^{13}$ vg/kg yielded circulating F.IX levels of 5-7% in the mice, and only 1-2% in hemophilic dogs $^{1,2}$. The reasons for this decline in efficacy with increasing size were not entirely clear; at the time, we speculated that inter-species differences in promoter activity (i.e. a cause not related to size), or diffusion distance of the product to the circulation (more clearly related to size of organism) might account for the difference. The diffusion distance of the vector in solid tissue is fairly constant. As the radius of the muscle bundle increases with increasing size of the animal, a lower fraction of the muscle is covered per injection. Thus, direct IM injection transduces large areas of muscle in a mouse, but relatively much less in a larger animal. Put simply, two injections of AAV-lacZ turn all muscles blue in a mouse but certainly not in a dog. To achieve scale-independent dosing, one needs to take advantage of a delivery technique based on fractal rather than Euclidean geometric principles. Regional intravascular delivery, utilizing the vascular tree of the isolated limb as the delivery network, affords such a solution to the problem of scaleable delivery.
The importance of this finding for gene delivery in hemophilia is considerable; this delivery method essentially closes the 10-50-fold gap in efficacy between liver-directed and muscle-directed AAV-mediated gene transfer in large animals\(^1,3^8\). For a disease category in which a substantial percentage (>80%) of severely affected adults have infection with hepatitis C\(^3^5,3^6\), it is crucial to have a treatment strategy that does not require the liver as the target organ for transduction.

Several investigators have shown that, in mice at least, AAV serotypes 1, 6, and 7 result in much more efficient transduction of skeletal muscle\(^4^1-4^3\). Confirmation of these results in large animal models has recently been published\(^8,4^4,4^5\). We were able to show in the hemophilia B dog model that direct intramuscular injection of AAV-1-canine F.IX resulted initially in high level F.IX expression, but was rapidly followed by inhibitor formation that, in contrast to isolated limb perfusion with AAV-2, could not be blocked by transient immunosuppression\(^8\). Others have also observed the formation of antibodies to the transgene product after intramuscular injection of more potent AAV serotypes\(^4^4,4^5\).

It would be of interest in their case to determine whether the transient immunosuppressive regimen that we employed could block antibody formation in their model system. All of our findings to date are consistent with the notion that high level expression of F.IX in the skeletal muscle of an animal not tolerant to the transgene product will, regardless of route of administration, trigger formation of inhibitory antibodies unless transient immunosuppression is administered\(^4\). Also translational studies in large animal disease models may uncover problems not appreciated in inbred strains of laboratory mice.
The data presented here, combined with data already in hand from earlier clinical studies, argue that this procedure can be safely scaled up to humans. We have documented the safety of administering AAV-2-F.IX vector doses up to $2 \times 10^{12}$ vg/kg to skeletal muscle by direct intramuscular injection in humans with hemophilia, and have shown, by immunohistochemistry on injected muscle tissue, that expression persists unabated at time points up to 10 months after vector injection. In other clinical studies, we have infused vector doses up to $5 \times 10^{12}$ vg/kg into the hepatic artery without systemic symptoms or serious adverse events. The surgical procedure outlined here, isolated limb perfusion, has been extensively used clinically for the delivery of high dose chemotherapy to a single limb. A recently described alternative to ILP is isolated limb infusion, a simplified less invasive technique for regional delivery of drugs through percutanenous catheterization of femoral vessels. Thus the safety of many individual elements of the treatment strategy is already established in humans. An important safety consideration for the procedure we described is the biodistribution of vector following intravascular delivery to skeletal muscle. We documented limited hematogenous dissemination of vector following ILP and in tissues harvested 8 weeks post procedure vector detection was limited to the perfused muscle. The use of a tight tourniquet and the removal of residual vector from the isolated vessels prior to reconnection to the systemic circulation are likely to restrict vector dissemination.

The current work demonstrates the efficacy of this delivery procedure in a large animal model of hemophilia B. Comparing the data generated here to results obtained in the
same animal model with the same vector delivered by direct IM injection \(^1\), it can be seen that the change in method of administration improves expression by as much as one log (compare circulating levels of \(~1\%\) after \(3.4 \times 10^{12}\) vg/kg by IM injection, to 4-15\% after ~2-4 \(\times 10^{12}\) vg/kg by ILP). These levels are especially encouraging since the recent studies in liver suggest that doses required for efficacy in the hemophilic dogs were predictive of doses required for efficacy in humans\(^37\). Moreover, this robust level of expression in the dog model allows margin for some loss of efficacy on translation to humans. The use of alternate serotypes which transduce skeletal muscle more efficiently in mice \(^8,^{41,42}\) may extend this margin if validity of the observation can be extended to large animals and humans. Although it will be necessary to address details of the immune response to the transgene product, and to identify an approved pharmacologic agent that can induce a vascular leak (i.e., a drug other than histamine), the studies here establish the efficacy of the strategy and thus permit us to build on the promising findings from the earlier AAV-F.IX study \(^7\) in which vector was administered by direct IM administration.
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COMPETING INTEREST STATEMENT

The authors declare that they have no competing financial interests.
REFERENCES:
Table I: Summary of canine F.IX antigen and clotting activity in hemophilia B dogs following delivery of AAV2-CMV-cF.IX by isolated limb perfusion (ILP) or by intravenous injection (IV)

<table>
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<th>Age at time of injection (months)</th>
<th>Gender</th>
<th>Weight (kg)</th>
<th>Route</th>
<th>Vector genome/kg</th>
<th>Circulating cF.IX (ng/ml)</th>
<th>aPTT* (seconds)</th>
<th>F.IX* activity (%)</th>
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<td>41 ± 3</td>
<td>1± 0.4</td>
<td>no</td>
<td>37</td>
</tr>
</tbody>
</table>

aPTT: activated partial thromboplastin time (normal dogs: $16.5 \pm 3.0$ seconds; hemophilia B dog >50 seconds)

* Values represent data from several time points (D99 and F57 from 2 to 26 months, H08 from 2 to 8 months)

** Animal did not receive cyclophosphamide.
FIGURE LEGENDS

Figure. 1. Histology of normal dog muscle eight weeks after direct intramuscular injection of AAV-CMV-canine F.IX. (a) Anatomy of dog hindlimb, showing sites of biopsy. (b) Immunofluorescence staining for canine F.IX at injection site. Original magnification 100X. (c) Hematoxylin and eosin staining of same site, showing normal histology. N.B. Vacuoles within muscle fibers are due to freeze artefact. (d) Immunofluorescence staining of uninjected muscle in same limb.

Figure. 2. Histology of normal dog muscle eight weeks after intravascular vector delivery by isolated limb perfusion. (a) Anatomy of dog hindlimb showing sites of biopsy. (b-f) Immunofluorescence staining for canine F.IX at multiple sites as indicated, showing extensive positive staining. (g-h) Hematoxylin and eosin staining of same samples. Original magnification 100X.

Figure. 3. (a) Canine F.IX antigen levels and (b) activated partial thromboplastin times (aPTT) in plasma samples of hemophilia B dogs as a function of time after delivery of AAV-CMV-cF.IX. Dog D99 (•••) injected with 3.7 x 10^{12} vg/kg, dog F57 (•••) with 1.7 x 10^{12} vg/kg, and dog H08 (•••) with 3.0 x 10^{12} vg/kg, by isolated limb perfusion, accompanied by transient immunosuppression. Dog E59 (•••) was injected by peripheral vein with 2.9 x 10^{12} vg/kg. Dog E60 (O-O) injected at 3.9 x 10^{12} vg/kg by ILP, without immunosuppression. Dog B48 (•••) injected by direct intramuscular injection with 3.4 x 10^{12} vg/kg. Arrow denotes infusion of canine plasma, resulting in transient spike in cF.IX and drop in aPTT.

Figure. 4. Coagulation assays in E60, dog treated by ILP without immunosuppression. (a) canine F.IX antigen levels, (b) activated partial thromboplastin times (aPTT), and (c) Bethesda assay (titer of neutralizing antibody) as a function of time after vector injection. Dog E60 injected at 3.9 x 10^{12} vg/kg and did not receive immunosuppresion. Gray band in (b) denotes range of normal aPTT, line at 50 sec denotes hemophilic values.

Figure. 5. Immunofluorescence staining of muscle sections of the tibialis anterior of hemophilia B dogs injected two (dog F57) or three (dog D99) years earlier with AAV-CMV-canine F.IX by isolated limb perfusion. (a, d) Fluorescence of rhodamine (red) showing presence of canine F.IX in muscle fibers of ILP injected dogs F57 (a) or D99 (d). (b, e) Fluorescence of FITC (green) showing presence of heparan sulfate proteoglycan (HSPG) in fibers of tibialis anterior of dog F57 (b) or D99 (e). (c, f) Simultaneous excitation of both fluorescence tags. (g) Fluorescence of rhodamine showing absence of canine F.IX expression in muscle biopsy from dog E59 (infusion of vector by peripheral vein). (h) Hematoxylin and eosin staining of same samples of dog (F57). Original magnification 100X.
Figure 1

a

d

b, c

b

NEGATIVE CONTROL

c

d
Figure 2

(a) Diagram of leg with labels:
- b
- c
- d
- e

(b) Image with green fluorescence

(c) Image with green fluorescence

(d) Image with green fluorescence and a white central line

(e) Image with green fluorescence

(f) Image with green fluorescence

(g) Image with pink staining

(h) Image with pink staining
Figure 3

(a) Graph showing the levels of aPTT (sec.) and eFIX (ng/ml) over time. Different symbols and lines represent various conditions or treatments.

(b) Graph showing the aPTT range with normal and hemophilic ranges indicated.
Figure 4

a. cF.IX (ng/ml)

b. aPTT (sec.)

c. Bethesda Unit

Days
Figure 5

(a)  
(b)  
(c)  
(d)  
(e)  
(f)  
(g)  
(h)
Regional intravascular delivery of AAV-2-F.IX to skeletal muscle achieves long-term correction of hemophilia B in a large animal model

Valder R Arruda, Hansell H Stedman, Timothy C Nichols, Mark E Haskins, Mattew Nicholson, Roland W Herzog, Linda B Couto and Katherine A High