STIMULUS REPORT

blood advances

Complete correction of hemophilia B phenotype by FIX-Padua skeletal muscle gene therapy in an inhibitor-prone dog model

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Key Points

- Skeletal muscle–directed expression of FIX-Padua resulted in complete correction of HB phenotype in an inhibitor-prone dog model.
- Long-term immune tolerance to FIX is sustained over years upon multiple challenges with recombinant FIX protein in 2 HB models.

Introduction

Hemophilia B (HB) is an X-linked bleeding disease resulting from deficiency of factor IX (FIX). Gene therapy is an attractive strategy for hemophilia because modest increases in clotting factor levels are associated with phenotypic improvement. Clinical trials for HB using adeno-associated viral (AAV) vectors targeting the liver are encouraging, and long-term expression of FIX wild-type (WT) transgene product ranges from 2% to 7% in the normal range.[1,2] However, in all AAV liver–directed trials, an anti–AAV-capsid cellular immune response is a limiting safety concern and seems to be vector-dose dependent.[3,4] Recently, the use of AAV-FIX-Padua (R338L) resulted in approximately fivefold higher FIX activity levels (~30% normal) at a four- to 10-fold lower vector dose[5] compared with AAV liver trials with FIX-WT,[1,2] while minimizing the risk of cellular immune responses. Transient immunosuppression is effective in controlling this complication in most, but not all, studies.[3,4] Moreover, adult hemophilia patients with underlying viral hepatitis from contaminated blood products[6] are ineligible for liver gene therapy; thus, alternatives to liver gene therapy are needed.

We have explored AAV delivery to skeletal muscle,[7-13] an ectopic tissue that generates biologically active FIX. HB dog models have proven highly informative with regard to the immune responses toward the transgene. Using HB dogs with low risk of inhibitor formation to canine (c) FIX (resulting from an *F9* missense mutation[14] from the University of North Carolina at Chapel Hill [UNC-CH]), we showed that intramuscular (IM) delivery to skeletal muscle of AAV-cFIX-WT had an excellent safety profile without a sustained anti-cFIX immune response.[7] However, similar IM injection protocol in inhibitor-prone dogs (resulting from an early stop codon *F9* mutation[15] from the University of Alabama at Birmingham [UAB]) resulted in inhibitor formation.[8] Thus, in the first clinical trial evaluating IM injection of AAV-FIX-WT, we restricted enrollment to subjects with missense mutations.[9] Though this therapy did not increase circulating FIX levels, there were no safety concerns in HB men.[9]

In previous reports, AAV-cFIX was delivered to skeletal muscle intravascularly in UNC-CH dogs with efficacious and safe outcomes.[10-12] We report here the first complete correction of the HB phenotype in the UAB model as well as the strength and duration of immune tolerance induction to the cFIX-Padua in both HB models.

Methods

AAV vector production and administration

Recombinant AAV serotype 6 vectors were produced by triple transfection[7] using an expression cassette containing cFIX-Padua under the control of cytomegalovirus promoter/enhancer (supplemental Figure 1).[12] Transvenular delivery to an isolated limb[10,16] and transient immunosuppression[17] has been described previously (supplemental Methods). The Institutional Animal Care and Use Committees at the Children's Hospital of Philadelphia, UAB, and UNC-CH approved all animal experiments.

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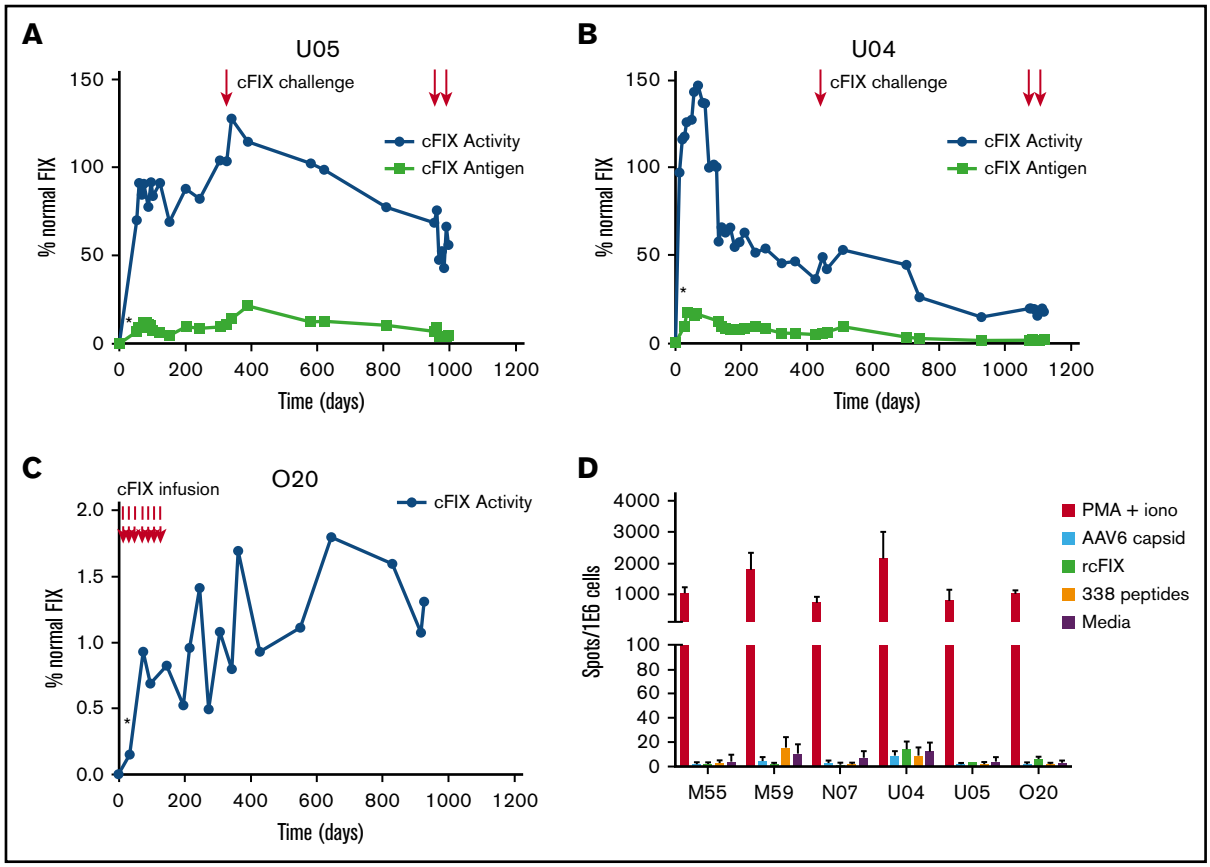

Figure 1. cFIX activity and antigen levels and cellular immune responses after peripheral transvenular delivery of AAV6-cFIX-Padua to skeletal muscle. Two adult inhibitor-prone UAB dogs ([A] U05, [B] U04) were treated with 3×10^{12} vg/kg, and 1 UNC-CH dog ([C] O20) was treated with 9×10^{11} vg/kg of AAV6-cFIX-Padua. cFIX antigen levels were measured by enzyme-linked immunosorbent assay (solid squares); cFIX activity levels were measured by activated partial thromboplastin time (solid circles). *Last dose of immunosuppression. Dashed arrows represent treatments with normal pooled canine plasma for bleeding; straight arrows represent immunogenic challenges with recombinant cFIX-WT. (D) Peripheral blood mononuclear cells collected 28 days after the last protein challenge were analyzed by interferon-γ ELISPOT analysis after stimulation with AAV6 capsid, cFIX-WT protein, or overlapping peptides spanning the 338 region (RATCLR/LSTKFTIYNM, LKVPVDRATCLR/LST). Phorbol myristate acetate plus ionomycin (PMA + iono) stimulation serves as the positive control. Bars indicate mean of 3 technical replicates and error bars are ± standard error of the mean.

Systemic toxicity

Complete blood counts, serum chemistries, and liver and kidney function were serially monitored.[10] Thrombin-antithrombin complex levels were measured using Enzygnost kit (Siemens).

rcFIX-WT protein production

Stable human embryonic kidney 293 cells expressing cFIX-WT were developed and recombinant cFIX-WT (rcFIX-WT) protein was purified with ion exchange chromatography. Dogs were challenged with 0.5 mg of cFIX-WT intravenously once per week for 1 to 2 weeks.

FIX expression, antibodies to FIX, and coagulation assays

The whole blood clotting time, cFIX antigen and activity levels, neutralizing antibodies to cFIX (Bethesda assay), and specific anti-c immunoglobulin G were measured as described.[10,18]

ELISPOT analysis

One-color enzyme-linked immunospot (ELISPOT) assays were used to measure interferon-γ T-cell responses to AAV-capsid, cFIX protein, or overlapping peptides spanning the 338 region of the cFIX protein.[18]

AAV-capsid NAb assays

Antibodies to AAV-capsid were assessed by enzyme-linked immunosorbent assay[19] and neutralizing antibodies (NAbs) by in vivo assays (supplemental Methods).[20]

Results and discussion

We report here the safety and efficacy of skeletal muscle expression of cFIX-Padua in UAB HB dogs. In this model, a single injection of cFIX-WT protein concentrate results in the formation of inhibitors against cFIX.[8] Intravascular delivery of AAV6-cFIX-Padua resulted in progressive increases in cFIX levels, with plateau activity levels of 54 ± 7% and 85 ± 20% of normal 150 days following vector administration (Figure 1A-B), with the seven- to 10-fold increased transgene specific activity[12,19] and normalization of their whole blood clotting time (Table 1). No bleeding events have occurred in ~6 years of cumulative follow-up. There was no local or systemic toxicity, no evidence of abnormal activation of coagulation (normal thrombin-antithrombin levels, data not shown), and no

Table 1. Summary of results in inhibitor-prone UAB HB dogs after skeletal muscle delivery of AAV6-cFIX-Padua (3×10^{12} vg/kg)

			Plateau* cFIX expression			Immune response			Bleeds/mo	
							IFN-γ ELISPOT			
Dog	Age/sex/weight	WBCT†, min	Activity, %	Antigen, %	Activity:antigen ratio	Anti–cFIX-IgG	cFIX	338 Peptides	Pretreatment	Posttreatment
U04	1 y/F/9.4 kg	10.8	54 ± 7	7.5 ± 2	7.6 ± 2	ND	ND	ND	8/13	0/37
U05	1.5 y/F/5.6 kg	10.5	85 ± 20	9.3 ± 5	10.4 ± 3	ND	ND	ND	1/17	0/33
								Total	9/30	0/70

F, female; IFN, interferon; ND, not detected; WBCT, whole blood clotting time.
*Plateau levels are averaged values after day 150. Values are mean ± standard deviation.
†Whole blood clotting time (normal values, 8-12 min and >60 min for untreated HB dogs).

clinical evidence of thrombosis. Interestingly, the cFIX levels in the UAB dogs are substantially higher than UNC-CH dogs that received identical vectors at similar doses and route of administration.[12] There were no differences between the AAV NAb titers before vector administration (supplemental Figure 2) nor the cFIX-Padua sera gene copy number (supplemental Figure 3A). The only notable difference was that the more tapered shape of the thigh of the UAB dogs was more conducive to the tourniquet, which likely leads to more effective vector delivery compared with the UNC-CH model (supplemental Table 1; supplemental Figure 4). Sex influences liver-directed AAV gene therapy, with lower efficacy in female compared with male mice, but not in skeletal muscle–directed.[21]

The strength of immune tolerance induction was confirmed by multiple challenges with rcFIX-WT protein. There was no formation of antibodies to cFIX despite widely spaced challenges even >2 years after stopping immunosuppression (supplemental Figure 5), nor was there a cellular response to cFIX protein or AAV6-capsid (Figure 1D). All dogs developed humoral response to AAV6-capsid (supplemental Figure 3B), which demonstrates their immunocompetent status. Thus, a comprehensive evaluation showed no immune response to FIX-Padua in a highly provocative scenario, combining the most challenging animal model[8] with a target tissue that is not predisposed to immune tolerance.[22]

Moreover, 1 UNC-CH dog (O20) received an inadvertent subtherapeutic dose of the same vector (Figure 1C; supplemental Figure 5C). This resulted in FIX antigen levels below the limit of detection (~0.3%), but sustained FIX activity of ~1% normal. Before reaching 1% FIX activity, O20 had 14 bleeds in 15 months, but subsequently, he did not bleed (31 months of observation). The complete amelioration of O20's bleeding phenotype with 1% activity demonstrates the in vivo hemostatic efficacy of FIX-Padua. Despite low antigen levels, O20 did not develop antibodies to cFIX, even after multiple administrations of canine plasma (containing cFIX-WT) to treat bleeds. We also challenged 2 UNC-CH dogs expressing cFIX-Padua from skeletal muscle[12] with rcFIX-WT >8 years after AAV delivery (supplemental Figure 6). Again, there was no detectable anti-cFIX immune response. These results suggest that even minimal levels of uninterrupted antigen expression provided by gene therapy are sufficient for sustained immune tolerance. The potential safety concern that low antigen levels trigger FIX inhibitor formation in mice[23] is not supported by our data in outbred models.

Combined, these data narrow the gap between liver and skeletal muscle AAV gene therapy, in terms of efficacy, by demonstrating long-term expression of curative levels of FIX-Padua, and in terms of safety, because there is no increased immunogenicity upon challenges. Moreover, the intravascular delivery method has already been proven safe in subjects with muscular dystrophy receiving saline alone[24] and would be anticipated to have a safer profile in HB subjects with healthy muscle tissue. Intravascular delivery of AAV to skeletal muscle may also overcome the inhibitory effect of NAb to vector capsid, as shown in preclinical models on vector readministration and prophylactic plasmapheresis.[10,25] Together, these data form the basis for translational studies for muscle-directed expression of FIX-Padua in patients with iatrogenic liver disease with a broad range of underlying *F9* mutations.

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Authorship

Contribution: R.A.F. executed the experiments of the phenotype assessment; B.J.S.-J. generated the recombinant canine proteins and helped drafted the manuscript; G.P.N. and C.D.L. performed the vector administration and provided care to the animals from University of Alabama at Birmingham; E.P.M. and T.C.N. performed the vector administration and provided care to the animals from University of North Carolina at Chapel Hill and protein challenges to all dogs; and V.R.A. directed experimental design, conducted data analysis, and interpretation and drafted the manuscript.

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