Brief report

The efficacy and the risk of immunogenicity of FIX Padua (R338L) in hemophilia B dogs treated by AAV muscle gene therapy

Jonathan D. Finn,1 Timothy C. Nichols,2 Nikolaos Svoronos,1 Elizabeth P. Merricks,2 Dwight A. Bellenger,2 Shangshen Zhou,1 Paolo Simioni,3 Katherine A. High,1,4,5 and Valder R. Arruda1,5

1Children’s Hospital of Philadelphia, Philadelphia, PA; 2University of North Carolina at Chapel Hill, Chapel Hill, NC; 3University of Padua, Padua, Italy; 4Howard Hughes Medical Institute, Philadelphia, PA; and 5University of Pennsylvania Perelman School of Medicine, Philadelphia, PA

Studies on gene therapy for hemophilia B (HB) using adeno-associated viral (AAV) vectors showed that the safety of a given strategy is directly related to the vector dose. To overcome this limitation, we sought to test the efficacy and the risk of immunogenicity of a novel factor IX (FIX) R338L associated with ~ 8-fold increased specific activity. Muscle-directed expression of canine FIX-R338L by AAV vectors was carried out in HB dogs. Therapeutic levels of circulating canine FIX activity (3.5%-8%) showed 8- to 9-fold increased specific activity, similar to humans with FIX-R338L. Phenotypic improvement was documented by the lack of bleeding episodes for a cumulative 5-year observation. No antibody formation and T-cell responses to FIX-R338L were observed, even on challenges with FIX wild-type protein. Moreover, no adverse vascular thrombotic complications were noted. Thus, FIX-R338L provides an attractive strategy to safely enhance the efficacy of gene therapy for HB. (Blood. 2012;120(23):4521-4523)

Introduction

Hemophilia B (HB) is an X-linked bleeding disorder resulting from a deficiency of coagulation factor IX (FIX). Gene therapy is an attractive strategy for the treatment of the disease because continuous maintenance of FIX levels as low as 1%-5% of normal have been shown to substantially ameliorate the bleeding phenotype in both preclinical and clinical models.1,6

Studies using adeno-associated viral (AAV) vectors showed that the safety profile is vector dose-dependent.3,4 In a liver-directed approach, immune responses to AAV-capsid proteins at the highest dose tested (2 × 1012 vg/kg) required transient immunosuppression for sustained transgene expression.3,4 In a study on direct intramuscular AAV-FIX, the safety profile was excellent2 and the local transgene expression of FIX in the injected muscle persisted for 3.7 and 10 years in 2 human subjects tested.7,8 However, all doses tested in the intramuscular study resulted in subtherapeutic circulating FIX levels.2 The use of FIX variants with gain of function offers the opportunity to enhance the efficacy of gene-based approaches for HB without increasing the vector doses. In an early study, we demonstrated that replacement of arginine 338 by alanine (R338A) was associated with an ~ 3-fold increase in the protein specific activity in murine models of HB receiving AAV-FIX-R338A9 as later confirmed in other models.10,11 Recently, we described a naturally occurring gain-of-function mutation in humans characterized by leucine at position 338 (R338L), which exhibits normal antigen levels, but an ~ 8-fold higher specific activity.12 Notably, the arginine at position 338 in FIX is conserved among mammals, and this region of the enzyme appears to be part of the substrate exosite for factor X.13 Here we report, for the first time, the use of the homologous FIX-R338L in a large and immunocompetent canine model of severe HB (< 1% of normal).

Methods

AAV vector production and administration

Recombinant AAV6 vectors were produced by a triple transfection protocol as previously described using an expression cassette containing canine FIX-R338L (cFIX Padua) under control of a cytomegalovirus promoter.1,2 The R338L mutation was generated using a QuickChange II-XL site-directed mutagenesis kit (Stratagene). All animal experiments were approved by the Institutional Animal Care and Use Committee at the Children’s Hospital of Philadelphia and the University of North Carolina at Chapel Hill. Three adult male HB dogs were administered between 2.5 and 3 × 1012 vg/kg of AAV6-cFIX-R338L by peripheral transvenous delivery to the skeletal muscle as previously described.1

Systemic and local toxicity

Hematologic and comprehensive biochemical analyses of blood and serum samples for liver and kidney function tests were performed as previously described.1,14 Thrombin/antithrombin complex (TAT) levels were measured at baseline and at day 50, 100, and >400 after AAV injection using Enzygnost TAT kit (Siemens Healthcare Diagnostic).

Normal plasma challenges

Two cFIX-R338L–expressing dogs (N07 and M59) were treated 4 times/wk with 100 mL of normal canine plasma intravenously. Plasma samples were collected before and after administration of the pooled normal plasma, and PBMCs were collected after the fourth challenge.

Canine FIX antigen, activity, and antibody assays

The whole blood clotting time, cFIX antigen and activity levels, neutralizing antibodies to cFIX (Bethesda assay), and non-neutralizing antibodies against cFIX were measured as previously described.1,14


There is an Inside Blood commentary on this article in this issue.

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ELISpot analysis

One-color ELISpot assays were used to measure IFN-γ T-cell responses to AAV capsid, cFIX protein, or overlapping peptides spanning the 338 region of the cFIX protein (RATCLRLSTKFTIYNM, LKVPPVRATCLRLST). All proteins and peptides were used at a final concentration of 10 μg/mL as previously described. Concanavalin stimulated PBMCs were used as a positive control, and media alone was the negative control. PBMCs collected after the fourth challenge with canine plasma were used for analysis.

Results and discussion

Skeletal muscle is an ideal target for the expression of therapeutic transgenes in genetic diseases that are associated with primary or iatrogenic underlying liver disease. In adults with hemophilia, the high rates of viral hepatitis resulting from blood transfusion preclude the enrollment of many patients in liver-directed gene therapy. We sought to test whether cFIX-R338L could be safely expressed in HB dogs by regional transvenular delivery of AAV vector to skeletal muscle of a single limb. We generated an AAV-6 vector encoding cFIX-338L and delivered it to 3 adult HB dogs. We chose to use an AAV-6 vector given the excellent tropism for muscle transduction in HB dogs1 and our own experience using AAV-6 for muscle transduction in HB dogs.1 There was a continuous increase in the circulating cFIX levels that reached plateau levels after day 100 (Figure 1). The kinetics and magnitude of expression in cFIX-338L dogs were similar to those observed in our earlier studies in dogs using cFIX wild-type (WT) via transvenular route. In a series of previous studies in HB dogs after liver, intramuscular, or intravascular delivery to skeletal muscle of AAV vectors of distinct serotypes expressing cFIX-WT, there was a linear correlation between cFIX antigen and activity levels (ration, 0.8-1.1). Here the ratio of antigen and activity clearly demonstrated that cFIX-338L exhibits an 8- to 9-fold higher specific activity of the protein (Table 1), as observed in humans with R338L. The expression of cFIX-338L was accompanied by normalization of the whole blood clotting time 1 week after vector delivery; this was stable throughout the follow-up (Table 1) and comparable with values observed in hemostatically normal dogs. Moreover, no spontaneous bleeding episodes requiring treatment occurred for a cumulative period of ~5 years after vector delivery (ongoing observations). The frequency of spontaneous bleeding episodes in untreated HB dogs is 5.5 bleeds per dog/year; thus, the phenotypic improvement supported by cFIX-338L is clinically evident.

One important consideration in any novel therapeutic approach for hemophilia is the risk of induction of neutralizing antibodies to the clotting factor. The success of gene therapy strategies to cure disease relies on the control of unwanted immune responses to transgene products, genetically modified cells, and/or to the vector. In early studies, we showed that transient immunosuppression (up to 4 weeks) of postskeletal muscle delivery of AAV-cFIX is required to prevent antibodies to the cFIX-WT transgene. One potential risk of introducing FIX with a variant sequence distinct from the WT protein is the immune response to the neoantigen product. The underlying mutation in the HB model

Table 1. Summary of results in hemophilia B dogs after skeletal muscle delivery of AAV-canine FIX R338L

<table>
<thead>
<tr>
<th>Dog</th>
<th>Age/sex/weight</th>
<th>Plateau cFIX expression</th>
<th>IFN-γ ELISpot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Activity, %</td>
<td>Antigen, %</td>
</tr>
<tr>
<td>M55</td>
<td>7 mo/male/23 kg</td>
<td>8</td>
<td>1.5</td>
</tr>
<tr>
<td>M59</td>
<td>7 mo/male/16.5 kg</td>
<td>3.5</td>
<td>0.35</td>
</tr>
<tr>
<td>N07</td>
<td>6 mo/male/13 kg</td>
<td>5.5</td>
<td>0.65</td>
</tr>
</tbody>
</table>

ND indicates not detected; and —, not done.

*Average ratio of activity/antigen ± SE from all time points.
†Whole blood clotting time: average values ± SE (normal values, 8-12 minutes).
from the University of North Carolina is a missense mutation that results in a G379E substitution in cFIX. Notably, there was no formation of inhibitory antibodies to cFIX-R338L on multiple challenges with cFIX-WT protein, even > 1 year after stopping immunosuppression (Figure 1B-C). These data were further supported by the lack of IFN-γ secretion by T cells after exposure to FIX-WT protein, peptides spanning the 338 residue with (R or L), and AAV-6 capsid (Figure 1D). Thus, the FIX-R338L variant shows no detectable immunogenicity in this approach. There was also no evidence of local or systemic toxicity, changes in TAT levels (data not shown), or clinically evident thrombotic complications; however, these data should be interpreted cautiously given the small number of animals used in this study. The latter is consistent with limited observation in humans where only patients with supraphysiologic levels of FIX-R338L (> 700% of normal) developed thrombosis, (and P.S., unpublished data, August 2011).

The expression of FIX-R338L variant has the potential to lower the therapeutic vector dose and is an attractive strategy to overcome limitations in performing critical posttranslational modifications in a variety of therapeutic settings, such as protein replacement or support the use of FIX-338L for clinical translational studies of HB.


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Authorship

Contribution: J.D.F. designed and executed the experiments and drafted the manuscript; T.C.N., E.P.M., and D.A.B. performed the vector administration, provided care to the animals, and collected laboratory samples; N.S. executed the T-cell assays; S.Z. provided the recombinant AAV vectors used in this study; P.S. and K.A.H. provided insights on experimental design and edited the manuscript; and V.R.A. directed experimental design, conducted data analysis and interpretation, and drafted the manuscript.

Conflict-of-interest disclosure: K.A.H. holds patents related to AAV manufacture and purification. She holds patents related to AAV-FIX, in which she has waived any financial interest. The remaining authors declare no competing financial interests.

Correspondence: Valder R. Arruda, Children’s Hospital of Philadelphia, 3501 Civic Center Blvd, 5056 Colket Translational Research Center, Philadelphia, PA 19104; e-mail: arruda@e-mail.chop.edu.
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