Bioengineered Factor IX Molecules with Increased Catalytic Activity Improve the Therapeutic Index of Gene Therapy Vectors for Hemophilia B

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Abstract

Although the desire to develop gene therapy for hemophilia B is high, safety remains a concern. Therefore, improving the therapeutic index of gene therapy vectors is an important goal. Thus, we evaluated the use of three bioengineered factor IX (FIX) variants with improved catalytic activity in the context of the helper-dependent adenoviral vector. The first vector expressed R338A-FIX, an FIX variant with the arginine at position 338 changed to an alanine, which resulted in a 2.9-fold higher specific activity (IU/mg) compared with the wild-type FIX. The second vector expressed FIX_{VIIIEGF1}, a variant with the EGF-1 domain replaced with the EGF-1 domain from FVII, which resulted in a 3.4-fold increase in specific activity. The third expressed R338A + FIX_{VIIIEGF1}, a novel variant containing both aforementioned modifications, which resulted in a 12.6-fold increase in specific activity. High-level, long-term, and stable expression of these three variants was observed in hemophilia B mice with no evidence of increased thrombogenicity compared with wild-type FIX. Thus, these bioengineered FIX variants can increase the therapeutic index of gene therapy vectors by permitting administration of lower doses to achieve the same therapeutic outcome. Furthermore, these variants may also be valuable for recombinant FIX protein replacement therapy.

Introduction

HEMOPHILIA B is an X-linked bleeding disorder characterized by a deficiency of coagulation factor IX (FIX). FIX is synthesized in the liver and circulates in the blood as a proenzyme. In the coagulation cascade, FIX is activated to FIXa by either contact activation via FXIa or as a consequence of vascular wall injury via tissue factor–FVIIa complex. FIXa then forms a complex with activated FVIII (FVIIIa) and this complex activates FX to FXa, which ultimately leads to the formation of a stable fibrin clot (Fig. 1). Current standard treatment for hemophilias is based on replacement of the deficient factor with frequent intravenous injections of recombinant protein. This therapy has been shown to be effective in limiting acute bleeds and reducing mortality and morbidity (Manco-Johnson *et al.*, 1994; Lofqvist *et al.*, 1997). However, current replacement is unsatisfactory and presents several problems including complications in frequent venous access, product supply, inhibitor formation, allergic reactions, and thrombosis (Ragni, 2004). Given these disadvantages, he-

mophilia B is among the best disease candidates for gene therapy because a low level of FIX (1–5% of normal) is therapeutic and because both small (mice) and large (dog) animal models are available for evaluation of novel therapies. However, vector-mediated toxicity remains an important concern. One way to minimize vector-mediated toxicity is to reduce the vector dose. This may be accomplished by using bioengineered FIX variants with higher catalytic activity so that the same therapeutic outcome can be achieved with lower vector doses. Two such catalytically enhanced human FIX variants have been described: R338A-FIX and FIX_{VIIIEGF1}. Compared with the wild-type FIX, R338A-FIX has the amino acid residue at position 338 changed from an arginine to an alanine (Chang *et al.*, 1998), which results in ~3-fold increase in FVIIIa-dependent clotting activity compared with wild-type FIX as determined by the activated partial thromboplastin time (aPTT) FIX assay. Kinetic analyses revealed that the increased clotting activity was due to an increased K_{cat} (increased turnover) and a decreased K_m (increased affinity) for FX, the substrate for the FIXa–FVIIIa complex (step 3 in Fig. 1) (Chang

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FIG. 1. Simplified diagram showing the role of coagulation factor IX (FIX) in the clotting cascade. FIX is activated to FIXa by FXIa or tissue factor–FVIIa complex (step 1). FIXa forms a complex with FVIIIa (step 2) to activate FX to FXa (step 3), which ultimately leads to the formation of a fibrin clot. R338A-FIX enhances step 3 and FIX^{VIIIEGF1} enhances step 2. The novel double mutant R338A + FIX^{VIIIEGF1} is hypothesized to enhance both steps 2 and 3. TF, tissue factor.

et al., 1998). FIX^{VIIIEGF1} has the first epidermal growth factor-like domain (EGF-1) replaced with the EGF-1 domain from the human FVII (Fig. 2) (Chang *et al.*, 1997). FIX and FVII are both vitamin K-dependent coagulation factors with homologous structural domains, and the EGF-1 domains of FIX and FVII share high sequence homology. Both have the same number of residues (38 amino acids) and exhibit high homology at the amino acid level (66%), and the conserved cysteine residues are involved in disulfide bond formation. FIX^{VIIIEGF1} has at least a 2-fold increase in clotting activity compared with wild-type FIX as determined by the aPTT FIX assay (Chang *et al.*, 1997). Importantly, when FIX^{VIIIEGF1} was administered to hemophilia B dogs, a greater than 3-fold increase in clotting activity *in vivo* was observed compared with equivalent amounts of wild-type FIX, normalizing the whole blood clotting in the absence of thrombotic complications (Chang *et al.*, 1997). The biological half-life was also demonstrated to be equivalent to that of wild-type FIX. The increased activity of FIX^{VIIIEGF1} was shown to be due to a higher affinity for FVIIIa, with a K_d about an order of magnitude lower than that of recombinant wild-type FIX (step 2 in Fig. 1) (Chang *et al.*, 1997).

Because the mechanisms of enhancement of R338A and FIX^{VIIIEGF1} do not overlap (Fig. 1), we hypothesized that a

novel double mutant combining both the R338A-FIX and FIX^{VIIIEGF1} mutations will have even greater catalytic activity than either mutation alone. Furthermore, we hypothesized that the overall three-dimensional structure of the protein will not be grossly altered by combining these two subtle mutations. Therefore, we have constructed this novel double mutant, which we have named R338A + FIX^{VIIIEGF1}, and have evaluated it for hemophilia B gene therapy.

In terms of gene therapy, expression of a catalytically enhanced FIX molecule from a vector would be highly beneficial because it would permit administration of lower vector doses to achieve the same therapeutic effect compared with the same vector expressing the wild-type molecule, thus increasing the therapeutic index. Likewise, for protein replacement therapy, lower amounts of the catalytically enhanced FIX molecules would be required for therapeutic benefit compared with the wild-type molecule, thus potentially reducing the cost significantly to perhaps permit the more desirable and effective prophylactic treatment regimen. In this study, we have evaluated the gene therapy potential of these three FIX variants in the context of the helper-dependent adenoviral vector (HDAd). HDAds are devoid of all viral sequences and thus are attractive for hemophilia B gene therapy because they can mediate long-term transgene expression in hemophilia B mice (Ehrhardt and Kay, 2002) and dogs (Brunetti-Pierri *et al.*, 2005a) without chronic toxicity.

Materials and Methods

Generation of bioengineered hFIX

Oligonucleotides 5'-GAC CGA GCC ACA TGT CTT GCA TCT ACA AAG TTC ACC ATC-3' and 5'-GAT GGT GAA CTT TGT AGA TGC AAG ACA TGT GGC TCG GTC-3' were used to generate the arginine-to-alanine modification in R338A-FIX and R338A + FIX^{VIIIEGF1}, using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotides 5'-GTG ACC AGT GTG CCT CAA GTC CAT GCC AGA ATG GGG GCT CCT GCA AGG ACC AGC TCC AGT CCT ATA TCT GCT TCT GCC TCC CTG CCT TCG AGG GCC GGA ACT GTG-3' and 5'-AGC TCA CAG TTC CGG CCC TCG AAG GCA GGG AGG CAG AAG CAG ATA TAG GAC TGG AGC TGG TCC TTG CAG GAG CCC CCA TTC TGG CAT GGA CTT GAG GCA CAC TG-3' containing the human FVII EGF-1 domain were hybridized, and used to replace the *Bst*EII–*Sac*I fragment containing the endogenous EGF-1 domain of human FIX to generate the FIX^{VIIIEGF1} modification. All cDNAs were sequenced to verify the desired modification.

Helper-dependent adenoviral vectors

All vectors were constructed in the pΔ21.7E4 backbone (Brunetti-Pierri *et al.*, 2005b) and they all contained an expression cassette composed of the following elements (from 5' to 3'): a liver-restricted rat phosphoenolpyruvate carboxykinase (PEPCK) promoter (Beale *et al.*, 1992), the apolipoprotein AI (ApoAI) intron, the human FIX cDNA, the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), the apolipoprotein E (ApoE) locus control region (LCR), and the human growth hormone polyadenylation signal sequence [p(A)]. HDAds were produced in 116 cells (Palmer and Ng, 2003) with the helper virus AdNG163 (Palmer and Ng, 2004) as described in detail elsewhere (Palmer and Ng, 2003). Helper

FIG. 2. HDAd containing wild-type (wt) FIX, R338A-FIX, FIX_{VIIIEGF1}, or R338A + FIX_{VIIIEGF1} expression cassettes. All expression cassettes contain the liver-specific phosphoenolpyruvate carboxykinase (PEPCK) promoter, the apolipoprotein AI (ApoAI) intron, the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), the ApoE locus control region (LCR), and the human growth hormone polyadenylation region [p(A)]. The adenoviral inverted terminal repeats (ITRs) and packaging signal (ψ) are shown. Not drawn to scale.

virus contamination levels were determined as described elsewhere (Palmer and Ng, 2003) and were found to be $<0.05\%$ for all vector preparations. DNA analyses of HDAd genomic structure were confirmed for both vectors as described elsewhere (Palmer and Ng, 2003).

Animal experiments

Animal procedures were approved by the Institutional Animal Care and Use Committee. Animals used for the experiments included the following: hemophilia B (FIX knockout) mice (Wang *et al.*, 1997) and C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME). For intravenous vector administration, retro-orbital injection was performed in hemophilia B mice (8–12 weeks old) at a dose of 1×10^{12} vector particles (VP)/kg. For the studies assessing thrombotic risk, C57BL/6 mice were injected at a dose of 5×10^{12} VP/kg. Statistical analyses were performed with the *t* test.

FIX antigen and FIX activity

Plasma human FIX antigen levels were measured by enzyme-linked immunosorbent assay (ELISA) (Enzyme Research Laboratories, South Bend, IN) according to the manufacturer's protocol. FIX activity was measured by the one-stage clotting assay, using human FIX-deficient plasma. The percentage of activity in plasma was determined from a calibration curve prepared with pooled plasma from C57BL/6 mice ($n = 5$). The aPTT reagent used was STA PTT Automate 5 (Diagnostica Stago, Parsippany, NJ) and was measured with an STart 8 hemostasis analyzer (Diagnostica Stago). FIX-deficient plasma was purchased from Precision BioLogic (Dartmouth, NS, Canada).

Markers of thrombus formation

Quantitative assessment of D-dimer levels in plasma was performed by an immunoturbidimetric method (Diagnostica

Stago). In this method, a beam of monochromatic light is allowed to traverse a suspension of microlatex particles to which specific antibodies have been attached by covalent bonding. In the presence of D-dimers, the antibody-coated latex particles agglutinate to form aggregates causing an increase in light absorbance. This increase in light absorption is a function of the antigen level present in the test sample. Using chromogenic methodology (Diagnostica Stago), quantitative antithrombin levels are measured by a two-step procedure: first, the plasma is incubated with a known excess of thrombin in the presence of heparin; second, the residual thrombin is quantitated by its amidolytic action on the synthetic chromogenic substrate CBS 61.50. The antithrombin level of the tested sample is inversely proportional to the residual thrombin in the second reaction step.

Results

Factor IX antigen levels and duration of expression

Hemophilia B mice ($n = 5$ or 6) were injected with HDAd expressing the bioengineered FIX molecules or the wild-type FIX at a dose of 1×10^{12} VP/kg. Two, four, thirty-six, and forty-four weeks postinjection, the levels of plasma FIX antigen were determined for each mouse (Fig. 3). Injection of all four vectors resulted in stable, high levels of FIX (Fig. 3). These results indicate that the bioengineered FIX variants do not compromise the duration of expression, suggesting that they are no more immunogenic than the wild-type FIX, at least in C57BL/6 hemophilia B mice.

Specific activity

We hypothesized that for a given dose, vectors expressing the bioengineered FIX molecules (R338A-FIX, FIX_{VIIIEGF1}, and R338A + FIX_{VIIIEGF1}) will result in higher specific activities (IU/mg) than the vector expressing the wild-type molecule. To test this, the percentage of normal FIX activity was determined

FIG. 3. Duration of FIX antigen expression in hemophilia B mice after intravenous administration of HDAd (1×10^{12} VP/kg) encoding wild-type FIX, R338A-FIX, FIX^{VIIIEGF1}, or R338A + FIX^{VIIIEGF1}.

for each of the aforementioned injected mice and the specific activity (IU/mg) was calculated to permit direct comparison between the four different treatment groups. By comparing specific activity, variables such as slight differences in vector infectivity (which may account for the differences in FIX antigen levels despite identical vector doses) or the exact amount of plasma antigen can be eliminated. The results indicated that compared with the vector expressing wild-type human FIX, the vector expressing R338A-FIX was more efficacious, yielding 2.2-fold higher activity at 2 weeks ($p = 0.00039$), 3.3-fold higher at 4 weeks ($p = 9.7 \times 10^{-6}$), 3.6-fold higher at 36 weeks ($p = 1.1 \times 10^{-5}$), and 2.3-fold higher at 44 weeks ($p = 0.0012$) than FIX-specific activities from the vector expressing the wild-type FIX molecule (Fig. 4), for an

overall average increase of 2.9 ± 0.7 -fold over wild-type. In the case of the vector expressing FIX^{VIIIEGF1} the specific activity was 2.0-fold higher at 2 weeks ($p = 0.00032$), 4.8-fold higher at 4 weeks ($p = 5.8 \times 10^{-7}$), 3.7-fold higher at 36 weeks ($p = 0.0025$), and 3.3-fold higher at 44 weeks ($p = 0.00015$) than that of wild-type FIX (Fig. 4) for an overall average increase of 3.4 ± 1.1 -fold over wild type. In the case of the vector expressing the novel double modification, R338A + FIX^{VIIIEGF1}, the specific activity was 8.9-fold higher at 2 weeks ($p = 1.4 \times 10^{-9}$), 19.9-fold higher at 4 weeks ($p = 5.8 \times 10^{-6}$), 16.7-fold higher at 36 weeks ($p = 4.3 \times 10^{-6}$), and 8.1-fold higher at 44 weeks ($p = 6.8 \times 10^{-6}$) than that of wild-type FIX (Fig. 4) for an overall average increase of 12.6 ± 4.8 -fold over wild-type. These results confirm that the single modifications (R338A and FIX-

FIG. 4. Specific activity (IU/mg) in hemophilia B mice 2, 4, 36, and 44 weeks after intravenous administration of HDAd (1×10^{12} VP/kg) encoding wild-type FIX, R338A-FIX, FIX^{VIIIEGF1}, or R338A + FIX^{VIIIEGF1}. Shown are averages $\pm$ SD ($n \geq 5$).

FIG. 5. (A) Plasma FIX activity and (B) plasma FIX antigen levels in hemophilia B mice 2 weeks after intravenous administration of HDAd (1×10^{11} VP/kg) encoding either wild-type FIX or R338A + FIX_{VIIIEGF1}. Shown are averages $\pm$ SD ($n = 3$ or 4).

FIX_{VIIIEGF1}) have significantly higher catalytic activity than the wild-type and suggest that they may improve the safety and efficacy of HDAd by permitting the use of lower vector doses to achieve the same therapeutic outcome. Furthermore, these results also indicate that the novel double modification (R338A + FIX_{VIIIEGF1}) has an even higher catalytic activity than each single mutant alone and may thus increase the therapeutic index of the vector even further.

To confirm this, hemophilia B mice ($n = 3$ or 4 per group) were injected with a vector encoding either the wild-type FIX or R338A + FIX_{VIIIEGF1} at a 10-fold lower dose (1×10^{11} VP/kg) as compared with the previous experiment. Two weeks post-injection, the plasma FIX antigen level and the percentage of normal FIX activity were determined. Mice injected with the vector encoding R338A + FIX_{VIIIEGF1} had $218.7 \pm 30.7\%$ of normal FIX activity (2.2 IU/ml) whereas mice injected with the vector encoding wild-type FIX had only $41.8 \pm 5.9\%$ normal FIX activity (0.42 IU/ml) (Fig. 5A). This 5.2-fold greater FIX activity ($p = 8.4 \times 10^{-5}$) was achieved despite the fact that the amount of FIX antigen was 1.6-fold higher ($p = 0.033$) in the mice injected with the vector expressing wild-type FIX (1192 ± 242 ng/ml) compared with the mice injected with vector encoding R338A + FIX_{VIIIEGF1} (742.7 ± 112.7 ng/ml) (Fig. 5B). Thus, these results confirm that greater therapeutic benefit can be obtained with a lower dose of the vector expressing R338A + FIX_{VIIIEGF1} compared with wild-type FIX.

Thrombogenicity

One concern regarding the use of catalytically enhanced FIX molecules is their potential to increase the risk for thrombotic complications. To assess for hypercoagulable state, we measured antithrombin III, fibrinogen, and D-dimer levels in wild-type mice ($n = 3$) injected with HDAd (5×10^{12} VP/kg) expressing either wild-type FIX or one of the three bioengineered FIX variants. The higher dose was chosen to magnify

any thrombogenicity if present. Despite stable expression of supraphysiological levels of FIX for more than 1 year (data not shown), there were no significant differences ($p \geq 0.12$) in any of the three aforementioned parameters (Table 1), suggesting that the three bioengineered molecules were no more thrombogenic than the wild-type molecule.

Discussion

In this study, we have sought to increase the safety and efficacy of hemophilia B gene therapy by using gene transfer vectors that express catalytically enhanced FIX molecules, with the rationale that such variants would increase the vector therapeutic index by permitting therapeutic outcome with lower doses. This is important because vector-mediated toxicity is dose dependent (Brunetti-Pierri *et al.*, 2004). We have evaluated vectors expressing two previously described bioengineered FIX molecules, R338A-FIX and FIX_{VIIIEGF1}, and indeed have found 2.9- and 3.4-fold increases in specific activity, respectively, compared with a vector that expressed

TABLE 1. MARKERS OF THROMBOSIS

Vector ^a	Antithrombin III (%) ^b	Fibrinogen (mg/dl) ^c	D-dimers (μ g/ml) ^b
FIX	109.3 $\pm$ 4.6	201.7 $\pm$ 2.3	0.44 $\pm$ 0.07
R338A-FIX	100.3 $\pm$ 8.1	216.7 $\pm$ 16.9	0.49 $\pm$ 0.23
FIX _{VIIIEGF1}	109.7 $\pm$ 8.1	219 $\pm$ 15.6	0.34 $\pm$ 0.21
R338A + FIX _{VIIIEGF1}	105 $\pm$ 3	208 $\pm$ 5.5	0.35 $\pm$ 0.08

Abbreviation: FIX, factor IX; HDAd, helper-dependent adenovirus.

^aHDAd (5×10^{12} VP/kg) expressing the indicated FIX variant was injected into wild-type mice ($n = 3$). Means $\pm$ SD are shown.

^bMeasured 3 weeks after injection of vector.

^cMeasured 2 weeks after injection of vector.

wild-type FIX, after vector administration to hemophilia B mice. These levels of enhancement are consistent with those observed for the purified, recombinant bioengineered molecules (Chang *et al.*, 1997, 1998). R338A-FIX has been used in a gene therapy setting with AAV (Schuettrumpf *et al.*, 2005). In that study, a 6-fold enhancement in FIX-specific activity over the wild-type FIX was reported after intravenous injection. We do not understand why there is a difference in the fold increase in FIX-specific activity among the R338A-FIX molecules in our study and in the study by Schuettrumpf and colleagues (2005); however, it should be noted that the study that originally reported the R338A variant (Chang *et al.*, 1998) showed approximately three times greater clotting activity than the wild-type FIX, which is closer to our results than the results reported by Schuettrumpf and colleagues (2005). Regardless, we believe the difference is modest (less than an order of magnitude). In an effort to further increase the therapeutic index, we produced and evaluated a novel FIX variant that combined R338A-FIX and FIX^{VIIIEGF1} into a single molecule and have found a 12.6-fold increase in specific FIX activity. The increase in catalytic activity was confirmed with a lower vector dose, thus confirming that the double modification in the FIX molecule allows the use of a lower vector dose to achieve improved therapeutic outcome (Fig. 5B). The use of bioengineered FIX molecules with enhanced specific activity to treat hemophilia B patients would offer many advantages. In the gene therapy context, use of such molecules would greatly improve safety and efficacy by permitting the administration of lower vector doses to achieve therapeutic benefit. For example, the innate inflammatory immune response to Ad-based vectors is dose dependent, exhibiting a steep threshold effect (Morrall *et al.*, 2002; Brunetti-Pierri *et al.*, 2004). Therefore, any reduction in vector dose would greatly increase the vector therapeutic index. In the case of protein replacement therapy, reduced amounts of the bioengineered FIX molecules would be required to achieve hemostasis compared with the wild-type molecule, thus potentially reducing the patient's cost significantly and perhaps permitting a more desirable and effective prophylactic treatment regimen.

One concern regarding the use of catalytically enhanced FIX is the risk of increased thrombosis. However, the evaluation of antithrombin III, fibrinogen, and D-dimer revealed that the three variants were no more thrombogenic than the wild-type molecule, even when present at supraphysiological levels. Thus, we conclude that the three bioengineered FIX molecules are no more thrombogenic than wild-type FIX. This is perhaps not unexpected because the modifications of the three variants do not alter normal FIX regulation (Chang *et al.*, 1997, 1998). Consistent with our results, purified FIX^{VIIIEGF1} was not observed to result in any thrombotic complications when administered to hemophilia B dogs (Chang *et al.*, 1997).

Hemophilia B patients develop inhibitors (neutralizing anti-FIX antibodies) at a frequency of 1–3%, which is detrimental because exogenously administered FIX protein will no longer be effective in response to bleeds. Therefore, another concern with bioengineered FIX variants involves the potential for increased risk of inhibitor formation. However, expression of all three variants, like the wild-type, persisted for the duration of the observation period of at least 44 weeks at a dose of 1×10^{12} VP/kg and more than 1 year at a dose of 5×10^{12} VP/kg, indicating that the variants are no more immunogenic than the wild-type molecule, at least in C57BL/6 hemophilia B mice.

In summary, we demonstrated that intravenous delivery of HDAd encoding R338A, FIX^{VIIIEGF1}, and R338A + FIX^{VIIIEGF1} resulted in circulating levels of FIX with higher biological activity compared with wild-type FIX. These bioengineered FIX molecules, and in particular our novel R338A + FIX^{VIIIEGF1} variant, may improve the safety and efficacy of HDAd by permitting the use of lower vector doses to achieve the same therapeutic outcome. In addition, these bioengineered FIX molecules may be valuable for other vector systems as well as for recombinant FIX protein replacement therapy. In the hemophilia B liver-directed clinical trial with AAV vector, the fact that two subjects developed transient transaminitis suggests a direct relationship between the AAV dose and the levels of hepatotoxicity as a consequence of an adaptive cellular immune response against the transduced hepatocytes (Manno *et al.*, 2006; Mingozzi *et al.*, 2007). It has been suggested that reducing the AAV dose may help to evade this immune response (Mingozzi and High, 2007). Therefore, the bioengineered FIX variants with increased catalytic activity described herein may be useful for achieving therapeutic FIX activity with lower vector doses.

Acknowledgments

This work was supported by the National Institutes of Health (R01 DK069369 to P.N.; K99HL088692 to N.B.-P.) and the Texas Affiliate of the American Heart Association (0765032Y to N.B.-P.). The financial support of Telethon-Italy (Fellowship GFP04008) to N.B.-P. is gratefully acknowledged.

Author Disclosure Statement

No competing financial interests exist.

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Received for publication June 20, 2008;
accepted after revision February 5, 2009.

Published online: April 2, 2009.