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Long-term expression of canine FVIIa in hemophilia dogs

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Abstract

The traditional treatment for hemophilia has been by protein replacement. This is complicated by the development of inhibitory antibodies to the infused factor (Factor VIII [FVIII] or Factor IX [FIX]). High-dose infusion of recombinant activated Factor VII (rFVIIa) has a long track record of success in such patients but its short-half life limits its use in prophylaxis. We have developed an alternative strategy by continuous expression of activated FVII from a *transgene* that is introduced into the host by means of gene transfer. For this, we modified the FVII cDNA to introduce a cleavage site between the light and heavy chain that would generate a FVII molecule secreted in the two-chain, activated form. Using viral-mediated delivery and expression from a liver-specific promoter (or as a transgenic approach) we demonstrated the long-term hemostatic efficacy of this approach in hemophilic mice. Subsequently, we used the canine version of our modified FVII and via gene transfer, showed multi-year phenotypic correction in hemophilic dogs, clearly evident by the absence of spontaneous bleeds that are characteristic in this animal model. No adverse events were observed throughout the study. Remarkably, clinical benefit was also observed in one treated dog despite the lack of hemostatic effect by *in vitro* assays. Overall, the results in this large animal model of hemophilia indicate the potential of gene-based continuous expression of activated FVII as a therapeutic strategy for hemophilia or other coagulation defects currently treated by rFVIIa.

Keywords

Factor VIIa; canine; animal model; gene transfer; hemophilia

Introduction – technology of FVIIa gene transfer

Blood coagulation is a vital homeostatic mechanism that involves interactions between cellular and protein components aimed at the controlled cessation of bleeding. This complex and highly-regulated process is defective in patients that have a deficiency in FVIII (hemophilia A) or FIX (hemophilia B). Although protein replacement therapy is successful in such patients, it can be complicated by the development of antibodies against the infused factor (~30% and 5% of severe hemophilia A and B, respectively) [1]. Nonetheless, inhibitor patients can be successfully treated by bolus infusion of high-dose rFVIIa (90–120 µg/kg) every two hours (until bleeding ceases), temporarily raising the FVIIa concentration sufficiently to generate high enough levels of thrombin for hemostasis in the absence of

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Conflict of interest statement

The author would like to disclose no relevant conflicts of interest.

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FVIII or FIX. However, the major drawback of rFVIIa is the short half-life (~2.7h) that limits its use in prophylaxis. As an alternative strategy, we have designed a gene-based approach using a modified FVII cDNA that can be secreted in the two-chain, activated form, following *intracellular* cleavage. We hypothesized that delivery and expression of such a transgene from an appropriate tissue/organ such as the liver, would result in elevated levels of FVIIa in the circulation (physiological levels of human FVIIa are ~1% of FVII i.e. ~5 ng/ml), promoting hemostasis under hemophilic conditions. Pilot studies using human FVII suggested that the introduction of the tripeptide repeat Arg-Lys-Arg at the Arg¹⁵²-Ile¹⁵³ (Arg¹⁵-Ile¹⁶ in chymotrypsin numbering, the normal site of cleavage of FVII during activation of the extrinsic pathway), resulted in a secreted protease with activity similar to rFVIIa [2]. Due to the low affinity of human FVII/FVIIa to murine TF [3–5], we developed the analogous murine FVIIa (mFVIIa) transgene and introduced it to the liver of hemophilia B mice with an Adeno-associated viral (AAV) vector [2]. This resulted in long-term *in vivo* hemostatic correction as a result of continuous expression of mFVIIa. Moreover, using a transgenic approach, we demonstrated that levels of mFVIIa within 0.5–1.5 µg/ml were sufficient to correct the defective hemostasis *in vivo* but expression >2µg/ml resulted in premature mortality, in a FX-dependent fashion [6]. Clearly, the issue of safety as well as efficacy of this gene-based approach needed to be addressed in an animal model that mimics the human condition more closely than the mouse.

Continuous FVIIa expression in canine hemophilia

Although mouse models of hemophilia allow for a rapid and comprehensive development of potential gene-based treatments, elements of their genetics as well the phenotypic manifestation of their coagulation defects do not closely mimic the human clinical phenotype. In contrast, hemophilia A and B dogs are larger in size, outbred, have a longer lifespan and demonstrate well-documented spontaneous bleeds (5.5/year [7]), attributes that make them better suited for the evaluation of potential treatment modalities in humans. This has been clearly established for protein-based therapeutics [8–12], as well as gene-based approaches with FVIII or FIX [13–16]. Thus, this animal model is ideal for the further evaluation of efficacy and safety of a FVIIa gene-based therapy.

We generated the canine version of the engineered FVIIa transgene (cFVIIa), based on the published sequence [17] in order to avoid any species-specific incompatibilities that could affect hemostasis in the treated dogs. Following purification of recombinant cFVIIa, we demonstrated its biological activity using *in vitro* assays [18]. Subsequently, we generated an AAV serotype 8 vector directing the expression of cFVIIa from a liver-specific promoter. Gene delivery of cFVIIa was performed initially in a hemophilia B male dog that received a vector dose of 2.06×10^{13} vector genomes (vg)/kg via the portal vein, a dose similar to the therapeutic dose utilized in hemophilic mice with mFVIIa ([6] and P. Margaritis, personal communication). Vector administration resulted in an initial decrease of the whole blood clotting time (WBCT, a measure of global hemostasis) that was most likely attributed to the prophylactic normal dog plasma administration that follows this procedure in the first few days. Shortly after stopping prophylaxis, the WBCT slowly returned to almost pre-administration level where it remained for the duration of the observation (34 months). Levels of circulating cFVIIa, as measured indirectly by a clotting assay, did not change and remained within the physiological range (<0.5 µg/ml), as was the prothrombin time (PT). However, this dog exhibited only 3 bleeds within the first 8 months post vector infusion, none of which were spontaneous, and remained free of bleeds thereafter (34 months of total observation). This is a remarkable finding since, based on empirical data, one would expect to observe 15 spontaneous bleeds [7]. Although further confirmatory experiments will be required, this suggests that low-dose gene delivery of cFVIIa may have a clinical benefit and thus have a potential application in a setting of prophylaxis.

The results from the first AAV-treated dog prompted a dose escalation study in a cohort of 3 hemophilia A dogs, with vector doses of 6.25×10^{13} vg/kg (one dog, male) and 1.25×10^{14} vg/kg (two dogs, one male and one female). This resulted in long-term, AAV vector-derived expression of cFVIIa, as measured by antigen levels (average of ~ 2 μ g/ml from these 3 dogs) and PT (Table 1). Although the WBCT in this cohort was reduced but not normalized, thromboelastography analysis of the extrinsic pathway using whole blood from these three treated dogs showed a normal or near normal profile, an improvement from pre-infusion measurements. Importantly, as a clear indication of a phenotypic benefit, none of these treated dogs exhibited spontaneous bleeds in a cumulative 45 months of observation; the expected number of such bleeds in that period would be 21 (Table 1).

In lieu of our observations in mice with mFVIIa $>2\mu$ g/ml [6], all AAV-cFVIIa treated dogs were closely monitored using markers of perturbed coagulation (platelet counts, levels of D-dimer, thrombin-antithrombin complex and fibrinogen), and in all dogs were found essentially within their respective normal ranges throughout the course of the study. In addition, despite administration of such high vector doses, no evidence of hepatotoxicity was found. Lastly, the expression of cFVIIa did not elicit an immune response against this transgene (except for a transient antibody within the first 4 weeks post gene transfer in one hemophilia A dog).

Approaches for improved gene-based FVIIa hemostasis

Our experience with AAV-cFVIIa delivery in hemophilic dogs revealed a potential limitation since relatively high AAV vector doses were required to demonstrate a hemostatic effect in vivo and in vitro. Thus, future strategies will have to focus on improving the expression of FVIIa and/or its hemostatic activity. For instance, enhanced expression can be a result of an improved delivery vector and/or expression cassette that includes the promoter element(s), intron, as well as the codon optimization FVIIa transgene itself, strategies that have been effective in other gene transfer approaches [19,20]. In addition, next generation human FVIIa analogs with enhanced activity due to modifications in the catalytic domain [21,22] or its binding to the phospholipid membrane [23,24] have been described and evaluated in animal models [25,26] and in non-hemophilic patients [27]. Given the extensive sequence homology between human, mouse and canine FVII, it may be possible to generate variant FVIIa transgenes with similarly enhanced function, that can be systematically evaluated in a gene transfer setting in hemophilic mice and dogs. Although in the hemophilia dog, continuous expression of cFVIIa at $\sim 2\mu$ g/ml was safe, additional studies will be required to establish the safety profile of this gene-based approach in cases where additional pathologic states complicate the disease phenotype (such as inflammation). Clearly, the future development of a regulatable expression system will contribute to the safety of this approach.

Conclusions

In summary, continuous expression of canine activated FVII via gene transfer at levels of $\sim 2\mu$ g/ml is safe and results in long-term hemostasis with a clear phenotypic impact on the bleeding diathesis of the recipient hemophilic dogs. Successful improvements of this mode of therapy may bring this closer to a therapeutic human application that, like protein infusion of rFVIIa, may be applied to a broad range of coagulation defects. Moreover, the current data also raise the interesting possibility that even low-dose FVIIa gene transfer may result in a reduction of spontaneous bleeds. Thus, this approach may also have merit as a mode of prophylaxis.

Abbreviations

FVII	Factor VII
FVIII	Factor VIII
FIX	Factor IX
FX	Factor X
AAV	Adeno-associated virus
PACE	Paired basic Amino acid Cleaving Enzyme
PT	prothrombin time
aPTT	activated partial thromboplastin time
mFVIIa	murine FVIIa
cFVIIa	canine FVIIa
cFVII	canine FVII
vg	vector genome
WBCT	whole blood clotting time
hAAT	human α_1 antitrypsin
ApoE	Apolipoprotein E
TEG	thromboelastography

References

1. Leissinger CA. Inhibitor development in patients with hemophilia: an overview. *Semin Hematol* 2006;43 (2 Suppl 4):S1–2. [PubMed: 16690370]
2. Margaritis P, Arruda VR, Aljamali M, Camire RM, Schlachterman A, High KA. Novel therapeutic approach for hemophilia using gene delivery of an engineered secreted activated Factor VII. *J Clin Invest* 2004;7:1025–31. [PubMed: 15057309]
3. Janson TL, Stormorken H, Prydz H. Species specificity of tissue thromboplastin. *Haemostasis* 1984;14:440–4. [PubMed: 6532909]
4. Kadish JL, Wenc KM, Dvorak HF. Tissue factor activity of normal and neoplastic cells: quantitation and species specificity. *J Natl Cancer Inst* 1983;70:551–7. [PubMed: 6601203]
5. Petersen LC, Norby PL, Branner S, Sorensen BB, Elm T, Stennicke HR, Persson E, Bjorn SE. Characterization of recombinant murine factor VIIa and recombinant murine tissue factor: a human-murine species compatibility study. *Thromb Res* 2005;116:75–85. [PubMed: 15850611]
6. Aljamali MN, Margaritis P, Schlachterman A, Tai SJ, Roy E, Bunte R, Camire RM, High KA. Long-term expression of murine activated factor VII is safe, but elevated levels cause premature mortality. *J Clin Invest* 2008;118:1825–34. [PubMed: 18398505]
7. Russell KE, Olsen EH, Raymer RA, Merricks EP, Bellinger DA, Read MS, Rup BJ, Keith JC Jr, McCarthy KP, Schaub RG, Nichols TC. Reduced bleeding events with subcutaneous administration of recombinant human factor IX in immune-tolerant hemophilia B dogs. *Blood* 2003;102:4393–8. [PubMed: 12933577]
8. Brinkhous K, Sandberg H, Widlund L, Read M, Nichols T, Sigman J, Oswaldsson U, Schaub RG, Mikaelsson M. Preclinical pharmacology of albumin-free B-domain deleted recombinant factor VIII. *Semin Thromb Hemost* 2002;28:269–72. [PubMed: 12098087]
9. Brinkhous KM, Hedner U, Garriss JB, Diness V, Read MS. Effect of recombinant factor VIIa on the hemostatic defect in dogs with hemophilia A, hemophilia B, and von Willebrand disease. *Proc Natl Acad Sci U S A* 1989;86:1382–6. [PubMed: 2784006]

10. Brinkhous KM, Sandberg H, Garriss JB, Mattsson C, Palm M, Griggs T, Read MS. Purified human factor VIII procoagulant protein: comparative hemostatic response after infusions into hemophilic and von Willebrand disease dogs. *Proc Natl Acad Sci U S A* 1985;82:8752–6. [PubMed: 3936044]
11. Brinkhous KM, Shanbrom E, Roberts HR, Webster WP, Fekete L, Wagner RH. A new high-potency glycine-precipitated antihemophilic factor (AHF) concentrate. Treatment of classical hemophilia and hemophilia with inhibitors. *JAMA* 1968;205:613–7. [PubMed: 5695099]
12. Brinkhous KM, Sigman JL, Read MS, Stewart PF, McCarthy KP, Timony GA, Leppanen SD, Rup BJ, Keith JC Jr, Garzone PD, Schaub RG. Recombinant human factor IX: replacement therapy, prophylaxis, and pharmacokinetics in canine hemophilia B. *Blood* 1996;88:2603–10. [PubMed: 8839853]
13. Jiang H, Lillicrap D, Patarroyo-White S, Liu T, Qian X, Scallan CD, Powell S, Keller T, McMurray M, Labelle A, Nagy D, Vargas JA, Zhou S, Couto LB, Pierce GF. Multiyear therapeutic benefit of AAV serotypes 2, 6, and 8 delivering factor VIII to hemophilia A mice and dogs. *Blood* 2006;108:107–15. [PubMed: 16522813]
14. Mount JD, Herzog RW, Tillson DM, Goodman SA, Robinson N, McClelland ML, Bellinger D, Nichols TC, Arruda VR, Lothrop CD Jr, High KA. Sustained phenotypic correction of hemophilia B dogs with a factor IX null mutation by liver-directed gene therapy. *Blood* 2002;99:2670–6. [PubMed: 11929752]
15. Sarkar R, Mucci M, Addya S, Tetreault R, Bellinger DA, Nichols TC, Kazazian HH Jr. Long-term efficacy of adeno-associated virus serotypes 8 and 9 in hemophilia a dogs and mice. *Hum Gene Ther* 2006;17:427–39. [PubMed: 16610930]
16. Wang L, Calcedo R, Nichols TC, Bellinger DA, Dillow A, Verma IM, Wilson JM. Sustained correction of disease in naive and AAV2-pretreated hemophilia B dogs: AAV2/8-mediated, liver-directed gene therapy. *Blood* 2005;105:3079–86. [PubMed: 15637142]
17. Callan MB, Aljamali MN, Margaritis P, Griot-Wenk ME, Pollak ES, Werner P, Giger U, High KA. A novel missense mutation responsible for factor VII deficiency in research Beagle colonies. *J Thromb Haemost* 2006;4:2616–22. [PubMed: 16961583]
18. Margaritis P, Roy E, Aljamali MN, Downey HD, Giger U, Zhou S, Merricks E, Dillow A, Ezban M, Nichols TC, High KA. Successful treatment of canine hemophilia by continuous expression of canine FVIIa. *Blood* 2009;113:3682–9. [PubMed: 19109232]
19. Nathwani AC, Gray JT, Ng CY, Zhou J, Spence Y, Waddington SN, Tuddenham EG, Kemball-Cook G, McIntosh J, Boon-Spijker M, Mertens K, Davidoff AM. Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood* 2006;107:2653–61. [PubMed: 16322469]
20. Wu Z, Sun J, Zhang T, Yin C, Yin F, Van Dyke T, Samulski RJ, Monahan PE. Optimization of self-complementary AAV vectors for liver-directed expression results in sustained correction of hemophilia B at low vector dose. *Mol Ther* 2008;16:280–9. [PubMed: 18059373]
21. Persson E, Bak H, Olsen OH. Substitution of valine for leucine 305 in factor VIIa increases the intrinsic enzymatic activity. *J Biol Chem* 2001;276:29195–9. [PubMed: 11389142]
22. Persson E, Bak H, Ostergaard A, Olsen OH. Augmented intrinsic activity of Factor VIIa by replacement of residues 305, 314, 337 and 374: evidence of two unique mutational mechanisms of activity enhancement. *Biochem J* 2004;379:497–503. [PubMed: 14686879]
23. Harvey SB, Stone MD, Martinez MB, Nelsestuen GL. Mutagenesis of the gamma-carboxyglutamic acid domain of human factor VII to generate maximum enhancement of the membrane contact site. *J Biol Chem* 2003;278:8363–9. [PubMed: 12506121]
24. Nelsestuen GL, Stone M, Martinez MB, Harvey SB, Foster D, Kiesel W. Elevated function of blood clotting factor VIIa mutants that have enhanced affinity for membranes. Behavior in a diffusion-limited reaction. *J Biol Chem* 2001;276:39825–31. [PubMed: 11517221]
25. Lauritzen B, Tranholm M, Ezban M. rFVIIa and a new enhanced rFVIIa-analogue, NN1731, reduce bleeding in clopidogrel-treated and in thrombocytopenic rats. *J Thromb Haemost* 2009;7:651–7. [PubMed: 19175492]

26. Tranholm M, Kristensen K, Kristensen AT, Pyke C, Rojkjaer R, Persson E. Improved hemostasis with superactive analogs of factor VIIa in a mouse model of hemophilia A. *Blood* 2003;102:3615–20. [PubMed: 12869500]
27. Moss J, Scharling B, Ezban M, Sorensen TM. Evaluation of the safety and pharmacokinetics of a fast-acting recombinant FVIIa analogue, NN1731, in healthy male subjects. *J Thromb Haemost* 2009;7:299–305. [PubMed: 19138379]

Table 1
Summary of in vivo studies of continuous expression of canine FVIIa in hemophilic dogs

Mode of delivery	Transgene	Promoter/enhancer	Vector dose (vector genomes/kg)	Dog model	Gender (age in years at vector infusion)	Average antigen (µg/ml)	Hemostatic effect	
							In vitro	In vivo
AAV8 viral vector	cFVIIa	hAAT/ApoE	2.06E13	Hemophilia B	M (0.25)	<0.5	No change in PT No change in WBCT	No spontaneous bleeds (in 34 months; 15 expected bleeds *)
AAV8 viral vector	cFVIIa	hAAT/ApoE	6.25E13	Hemophilia A	M (0.9)	~1.8	↓ PT ↓ WBCT Near-normalization of TEG profile	No spontaneous bleeds (in 18 months; 8 expected bleeds *)
AAV8 viral vector	cFVIIa	hAAT/ApoE	1.25E14	Hemophilia A	F (1.2)	~2.0	↓ PT ↓ WBCT Normalization of TEG profile	No spontaneous bleeds (in 15 months; 7 expected bleeds *)
AAV8 viral vector	cFVIIa	hAAT/ApoE	1.25E14	Hemophilia A	M (6.5)	~1.8	↓ PT ↓ WBCT Near-normalization of TEG profile	No spontaneous bleeds (in 12 months; 6 expected bleeds *)

Total cumulative months of observation		Observed vs. expected spontaneous bleeds	
Hemophilia A	Hemophilia B	Hemophilia A	Hemophilia B
45	34	None vs. 21	None vs. 15

Single arrow (↓) indicates decrease relative to untreated animals; hAAT: human alpha1 antitrypsin; ApoE: apolipoprotein E enhancer (4 tandem copies); TEG: thromboelastography (extrinsic pathway);

* Based on empirical data [7]