Journal of Genetic Syndromes & Gene Therapy
ISSN: 2157-7412

Quade-Lyssy et al., J Genet Syndr Gene Ther 2012, S:1
DOI: 10.4172/2157-7412.S1-013

Review Article Open Access

Engineered Factor VII, Factor IX, and Factor X Variants for Hemophilia Gene Therapy

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Abstract

The coagulation factors VII, IX, and X are all vitamin K dependent serine proteases synthesized in the liver with a high degree of similarity concerning size and structure. Factor IX is the deficient protein in hemophilia B and used in substitution therapy. The activated form of factor VII (FVIIa) is used to treat hemophilia in which the coagulant tenase complex cannot form due to inhibitory antibodies predominantly directed against factor VIII (FVIII) in hemophilia A. For both FVIIa and FIX, diverse approaches in protein engineering have been successfully applied to enhance protein activity, secretion, or half-life. Approaches include modification of functional important amino acid residues as well as the generation of fusion proteins. FX, on the other hand is the common substrate of both FVII as well as FIX. More recently, engineering of FX was therefore employed to shortcut FX activation by the physiological tenase complex, which is affected in both disorders, hemophilia A and B. All three proteases might therefore play a role in gene transfer strategies for hemophilia. In early clinical studies, the expression levels following gene transfer have been limited by dose dependent immune responses against the vector (AAV, liver-directed) or the therapeutic transgene (AAV, intramuscular). Therefore, protein modification might provide the necessary improvement to lift therapies in the therapeutic range while limiting vector exposure and local expression levels. Shifting gene transfer from FVIII to FVIIa or to variants of FVIIa, FIX or FX might additionally be a strategy to bypass inhibitory antibodies against FVIII and address limits of the vector packaging due to size limit restrictions encountered in FVIII gene transfer at the same time.

Keywords: Hemophilia gene therapy; Coagulation factors; Amidolytic activity; Coagulation factors; Inhibitory antibodies

FVII/FVIIa

Patients with hemophilia A and B have deficient protein concentrations of FVIII and FIX, respectively and therefore are treated by protein substitution with plasma-derived or recombinant factor FVIII or FIX concentrates. In about 20-30% of the patients with hemophilia A and in 3-5% of those with hemophilia B this can lead to the development of neutralizing antibodies against the infused proteins which makes therapy inefficient [1,2]. Recombinant activated factor VII (rFVIIa) protein is currently an alternative therapy available for inhibitor patients to treat excessive bleeding. However, supraphysiological concentrations and repeated administration are required to induce hemostasis [3].

Consequently, FVIIa analogs were created for future protein replacement therapy or gene delivery approaches based on crystalline structure analysis of free and TF-bound FVIIa or on sequence homology of other more potent proteases [4,5].

Like the other vitamin K dependent coagulation factors, FVII is physiologically synthesized in the liver. FVII is secreted into the circulation as a 406-residue single-chain polypeptide which contains an N-terminal γ-carboxyglutamic acid (Gla) domain in which all ten Glu residues are post-translationally carboxylated followed by two regions homologous to epidermal growth factor domains and a serine protease domain [3,6,7]. Upon vascular injury, the cofactor tissue factor (TF) is exposed to FVII and triggers the extrinsic pathway of the coagulation cascade by activation of FIX, factor X and FVII auto-activation on the TF-bearing cells resulting in large-scale thrombin generation and a fibrin clot. FVII is activated to FVIIa by internal proteolysis due to a cleavage of a single Arg152-Ile153 peptide bond. The physiological plasma concentration of FVII is around 10 nM, however, only about 1% of FVII is circulating in its free and active form. FVIIa has a plasma half-life of approximately 2.5 hours [8]. FVII activation results in connected FVII light and heavy chains which form a one-to-one complex with TF in the presence of Ca^{2+} ions. The complex formation is an essential process in which TF allosterically leads to a fully active FVIIa [9]. The cleavage alone leaves FVIIa in a zymogen-like conformation of quite low specific activity [3,10]. Several residues in the first EGF-like domain of FVII and in the protease domain, especially methionine at position 306, seem to be pivotal for the cofactor-mediated allosteric stimulation [11]. TF stabilizes the active conformation of FVIIa for accelerated activation of its substrates FIX and FX [12,13] by inducing a conformational change and establishing a stable salt bridge between the residues Ile153 and Asp343 [14] and stabilization of the interaction between the residues Leu305 and Phe374 which in turn stabilizes S_1 and S_3 substrate pocket and the activation pocket [15]. These findings contributed to the development of FVIIa variants with enhanced TF-independent (intrinsic) specific activity by mimicking the effect of TF binding [16]. Additional findings (described below) focused on enhancing membrane affinity, a necessary component of procoagulant reactions [5]. Overall, such variants would be able to bypass the intrinsic

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Received October 30, 2012; **Accepted** December 07, 2012; **Published** December 10, 2012

Citation: Quade-Lyssy P, Milanov P, Schüttrumpf J (2012) Engineered Factor VII, Factor IX, and Factor X Variants for Hemophilia Gene Therapy. J Genet Syndr Gene Ther S1:013. doi:10.4172/2157-7412.S1-013

pathway and were beneficial agents for the treatment of hemophilia patients who already developed inhibitory antibodies.

Engineering FVIIa variants with increased intrinsic activity

Crystal structures of free and TF-bound FVIIa showed slight differences but indicated a rearrangement of α-helix consisting of residues 307-312 [7]. Following substitution of amino acids in vicinity to this region, namely Leu305Val and Phe374Pro, slightly increased amidolytic and proteolytic activity by 4-fold and 1.5-fold, respectively, mediating a local reorganization of the helix [10]. More potent variants were generated by introducing amino acids which can be found in thrombin possessing a considerable constitutive activity. The thrombin motif appeared in the variant $FVIIa_{IIa}$ containing the following mutations V158D/E296V/M298Q which led to an 8-fold higher intrinsic amidolytic activity and remained about 70% of its activity even in absence of calcium. The authors suggested that the mutations imitate the allosteric effect of TF because once the FVIIa-TF complex is built; it is independent of calcium ions and facilitates salt bridge formation with Asp343. Combination of $FVIIa_{IIa}$ with the exchange K377A could even enhance TF-independent amidolytic activity which was 11-fold higher than that of wild-type FVIIa and even increased substrate specificity. The latter was probably due to a shortening of the long lysine residue to alanine which seemed to facilitate access of substrate to the active site. Both events (salt bridge and K377A) resulted in a dramatic enhancement of FX activation und thus in a 25- and 50-fold higher thrombin generation for $FVIIa_{IIa}$ and K337A-$FVIIa_{IIa}$, respectively. Both variants exhibited an increased rate of inhibition by antithrombin in the presence of heparin which was seen also for L305V-FVIIa [16]. Subsequent analysis of each individual mutation of $FVIIa_{IIa}$ revealed that each position contribute to an improved property. E296V and M298Q are required for the increase in amidolytic activity and salt bridge formation with Asp343. E296V additionally reduces the calcium dependence and M298Q encloses an enhanced factor X activation [17]. Another promising FVIIa variant carried the mutations Leu305Val/Ser314Glu/Lys337Ala/Phe374Tyr ($FVIIa_{VEAY}$) which contributed to an increased stabilization of the 170 loop (helix-loop with residues 307-321) and an optimized helix orientation. This variant showed improvements over wild-type FVIIa with 22-times higher rates in intrinsic amidolytic activity, a 5.5-fold reduced K_m value and a 4-fold higher substrate turnover rate. Nevertheless, in comparison the variant $FVIIa_{IIa}$ had a 3-fold advantage over $FVIIa_{VEAY}$ in FX activation [4,18]. Tranholm et al. determined the *in vivo* effects of FVIIa variants following intravenous administration at doses of 1 and 3 mg/kg *vs.* rFVIIa at doses of 1, 3, 6, and 10 mg/kg. The following three FVIIa variants, M289Q-FVIIa, $FVIIa_{IIa}$ and K377A- $FVIIa_{IIa}$ were investigated in a tail-bleeding model in mice with antibody induced hemophilia A. The variants shortened the clotting times and reduced blood loss. Histopathological analysis of kidneys didn't reveal any changes like fibrin deposition. The variants showed a 3-4 times higher potency above rFVIIa with reduced doses (3 mg/kg *vs.* 10 mg/kg rFVIIa) at the same time [19]. However this was much less than observed *in vitro* [16]. It was also demonstrated that despite higher FXa generation the variants didn't initiate an excessive thrombin generation on the surface of unperturbed endothelium suggesting a reduced thrombogenic potential [20].

NovoNordisk focused more intensively on the variant $FVIIa_{IIa}$ (INN name is vatreptacog alfa, formerly designed as NN1731). *In vitro* studies of clot formation with $FVIIa_{IIa}$ in whole blood of hemophilia A patients with and without inhibitory antibodies to FVIII showed a dramatic decrease of clotting time compared to rFVIIa and was even shorter than the CT values of healthy males [21]. $FVIIa_{IIa}$ also improved fibrin formation, structure and stability in hemophilic plasma [22]. In a clinical phase I study the safety and pharmacokinetics were evaluated in 8 healthy males after administration of a single dose of vatreptacog alfa. The FVIIa analog was safe and well tolerated at doses up to 30 µg/kg without any evidence of adverse events [23]. The phase II clinical trial evaluated the safety and efficacy of vatreptacog alfa *vs.* rFVIIa (Novoseven®) to treat joint bleeds of hemophilia A and B patients with inhibitors. The study included 51 patients in which a total number of 96 joint bleeds were treated including 77 bleeds with vatreptacog alfa and 19 bleeds with rFVIIa. Overall, vatreptacog alfa was successfully in control of 98% of the joint bleeds compared with 90% of bleeds treated by rFVIIa. Dose-escalation (one to three doses at 5, 10, 20, 40 and 80 µg/kg *vs.* 90 µg/kg rFVIIa) showed that treatment success was already higher at 20 to 80 µg/kg with vatreptacog alfa. Also the number of doses needed to stop bleeding decreased with increasing dose of vatreptacog alfa. Overall, treatment was well tolerated with a low frequency of adverse events. In none of the treated patients antibody development could be detected and there was no thrombotic events related to the treatment [24]. These two clinical studies seemed very promising. Thus, a phase 3a clinical study (adept™2) was performed and completed by Novo Nordisk (Registration number: NCT01392547). 72 patients were involved to treat acute bleeding episodes in patients with congenital hemophilia and inhibitors with vatreptacog alfa *vs.* rFVII (NovoSeven). Treatment was provided on demand and stopped bleeding with 93% with three doses or less. Nevertheless, a number of patients developed antibodies against vatreptacog alfa. This phenomenon was not seen in treatment with rFVII (NovoSeven). It seems that replacement of three amino acids triggered the immune system to develop neutralizing antibodies against the drug. Therefore NovoNordisk decided to discontinue the development of vatreptacog alfa because of safety.

Engineering FVIIa variants with higher membrane affinity

A comparison study of vitamin K-dependent proteins focused on the correlation of amino acid substitutions and membrane binding properties. It was predicted that changing amino acid residues on positions 11, 33 and 34 (bovine prothrombin numbering) could change membrane affinity [5]. The first FVII variant with a 20-fold higher membrane binding contained the amino acid substitutions Gln for Pro10 and Glu for Lys32 (VII-Q10E32). Furthermore, this variant showed several changed characteristics over wild-type FVII including a 100-fold faster auto-activation and a 50-fold increased activity [25]. The evaluation of the already activated factor VII-Q10E32 (QE-VIIa) in comparison to activated wild-type FVIIa revealed an up to 40-fold higher function in both TF-dependent and TF-independent reactions [26]. Insertion of a tyrosine at position 4 and introduction of additional replacements at position 33 and 34 resulted in a FVIIa variant containing the following substitutions (Y4)P10Q/K32E/D33F/A34E and a 150-296-fold enhancement over wild-type factor VIIa under defined conditions [27]. It seemed that changing amino acid residues at the surface of the Gla domain could beneficially influence the membrane affinity.

Engineering FVIIa variants with prolonged half-life for gene therapy

The short half-life of FVIIa and the need of repeated intravenous injections to stop bleeding in patients with bleeding disorders triggered the interest in long-acting rFVIIa proteins. Half-life prolonged FVIIa could also advance gene therapy approaches as lower vector doses would be needed to obtain comparable stable circulating FVIIa levels.

One way to prolong half-life is genetic fusion of FVIIa and a protein with a longer half-life in circulation. Weimar et al. fused albumin to the C-terminus of rFVIIa via a flexible glycine-serine linker [28]. Albumin is the most abundant protein in plasma with a half-life of about 20 days. Although *in vivo* half-life of the fusion protein (rFVIIa-FP) was significantly lower than that of albumin, it showed its superiority to wild-type rFVIIa with a factor of 6.7 without compromising activity. The *in vivo* efficacy was confirmed in a rat FVII-depletion model. Clotting times were corrected to normal even 16 h after application of rFVIIa-FP indicating that the protein was still functionally active while recombinant FVIIa showed no more effects on clotting time due to its short half-life of 2.4 h [28,29]. Recently, a phase I clinical trial from CSL-Behring about safety and pharmacokinetics of intravenously infused rFVIIa-FP in healthy male volunteers was completed (Registration Number: NCT01542619) and also Novo Nordisk completed a phase 1 (Registration Number: NCT00922792) and phase 2 clinical study (Registration Number: NCT00951405) with a long-acting FVIIa analog. The data are not yet available but these studies will give us important information about expectable adverse events and antibody development of these variants concerning a gene therapy approach. Another *in vitro* study investigated the influence of the activation peptide on the plasma half-life of the coagulation factors VII, IX, X and protein C which share a similar protein structure. But within the protein family FVII has the shortest half-life and lacks an activation peptide while the half-life of protein C, FIX and FX increases with increasing length of the activation peptide. Insertion of the activation peptide from FX into the zymogen FVII resulted in a 4-fold half-life extension. The authors suggested that the activation peptide motif acts as plasma retention signal. The positioning of the FX activation peptide within the FVII molecule seemed not to be critical for prolongation of half-life but indeed affected the procoagulant activity. The location at the C-terminus of FVII led to a cleavable protein with normal amidolytic activity but with an impaired FX activation while the N-terminus located FX activation peptide diminished activation [30]. FVIIa variants with extended half-life may improve bypassing agents with a more reliable and sustained hemostatic efficacy for the treatment of acute bleedings in hemophilia. Until now, there are no existing preclinical gene therapy studies for long-acting FVIIa variants although such strategy seems to be feasible. It is also uncertain whether half-life extension would be a real benefit since gene therapy would provide continuous and long-term FVIIa expression. However, one would assume that higher circulating plasma levels of FVIIa could be achieved by gene therapy with such variants without the need to increase vector dose.

Preclinical FVIIa gene therapy studies

AAV-mediated gene transfer using a modified FVII expression construct, in which a paired amino acid cleaving enzyme (PACE)/furin intracellular cleavage site was introduced between the heavy and the light chain of FVII, resulted in direct secretion of the activated protease. Expression levels of the transgene reached up to 1000 ng/ml with the highest vector dose injected and didn't induce any thrombotic complications but still corrected phenotype [31,32]. In contrast, the overexpression at levels greater than 2 µg/ml caused mortality and pathological changes in heart and lung [33]. Gene transfer was also investigated in a large animal model (canine), which is a more related model for hemophilia and a good predictor of efficacy and safety of hemophilia treatments. Canine FVIIa was delivered to liver by a serotype 8 AAV vector via portal vein administration. Treatment resulted in long-term expression of cFVIIa ranging between 1.3 and 2.6 µg/ml. Prothrombin time and whole blood clotting time were shortened and treated dogs did no longer exhibit any spontaneous bleeding episode. There was also no evidence for hepatotoxicity or thrombotic events [34]. In a recent study two murine FVIIa variants based on the already described human FVIIa variants containing either the amino acid substitutions DVQ or VEAY, were used for viral AAV-based gene delivery for continuous FVIIa expression levels in a hemophilia mouse model. The variants showed a 6- to 17-fold increased proteolytic and intrinsic activity (in absence of TF) *in vitro*. Following gene delivery of $mFVIIa_{VEAY,}$ long-term and effective hemostasis was achieved at a considerably lower antigen expression (0.6 µg/ml *vs.* 3 µg/ml) due to reduced viral vector doses compared with mFVIIa even in the presence of inhibitors. However, supraphysiological expression levels of the variant (~8 µg/ml) resulted in increased TAT levels and led to a mortality of 70% in FVIII knockout mice within 6 weeks after vector administration. Immunohistochemical staining of heart and lung revealed ongoing clot formation in the microcirculation [35]. So far, preclinical studies of gene based therapy in mice or dogs showed that expression of FVIIa at physiological concentrations (up to 2 µg/ml) is efficient and safe. A combination of prolonged half-life and higher FVIIa activity could improve gene delivery of FVIIa allowing reducing vector dose. Therefore, the FVIIa variants show a great potential for the future treatment of inhibitor patients, however dosing of activated FVIIa seems to be critical in continuous expression. Although the clinical experience with FVIIa protein infusion indicates no safety concerns in hemophilia, continuous expression of an activated clotting factor might carry additional risks, which have to be addressed before moving into the clinics. To date, there is no clinical trial registered for a gene therapy approach with FVIIa variants.

FIX

Alike to FVII, factor IX is a clotting protein which is secreted from liver cells into the plasma as a 415-residue single chain molecule. Upon vessel injury FIX becomes activated through proteolytic cleavage at Arg180-Val181 and Ala146-Arg180 by FXIa and by FVIIa-tissue factor complex. The activated FIX serine protease (FIXa) can form together with its non-enzymatic cofactor FVIIIa and FX a macromolecular complex in which FX is activated. The FIX Gla domain comprises 12 N-terminal glutamic acid residues which are posttranslational γ-carboxylated [36]. Adjacent to the N-terminal Gla domain two EGF-like domains are followed by the heavy chain of FIX which encompasses the protease domain. Throughout the last decades several structural and functional analyses on coagulation factor FIX led to the development of various variants displaying both improved activity as well as enhanced protein secretion.

Engineering FIX variants with enhanced specific activity

A considerable increase in FIX activity through substitution of amino acid 338 from arginine to alanine was first reported by Chang et al. [37]. The R338A substitution which is placed in FVIIIa binding region within the FIX protease domain [38] resulted in 3-fold higher clotting activity compared to wild-type (wt)-FIX in presence of FVIII. Although cleavage at Arg338-Ser339 by thrombin has been proposed to act as possible inactivation mechanism in FIXa [39], thrombin inhibition by hirudin had no impact on clotting activity for either wt-FIXa or FIXa-R338A. Hence the observed improvement in catalytic activity was almost certainly not caused through elimination of a putative thrombin cleavage site at amino acid 338 [37]. The observed increase in catalytic efficiency was rather the outcome of the greater binding affinity of FIXa-R338A to FVIIIa [40]. Since those reports several independent studies were implemented in the bioengineering of new combined variants with the substitution R338A in order to

obtain an additive effect in catalytic activity [40,41] and thereby to succeed more efficacious gene therapy approaches by means of novel FIX variants with enhanced procoagulant potential for treatment of hemophilia B [40,42]. A further increase in FIX activity could be achieved by combining R338A with two other mutations (V86A and E277A) resulting in a FIX triple variant which exhibited 13-fold higher specific coagulation activity and 10-fold higher affinity for human FVIIIa [40]. Another group generated a similar increase in specific clotting activity (12.6 fold higher than wt-FIX) through replacement of the FIX-EGF-1 domain by the corresponding sequence of FVII supplementary to the R338A substitution [41]. Moreover, expression of FIX-R338A variants following liver-directed gene transfer led to an improvement in specific clotting activity and hemostasis [42] in hemophilia B mice following delivery of both adeno-associated viral (AAV) vectors [40,42] as well as helper-dependent adenoviral (HDAd) vectors [41]. Lastly, even one more efficacious variant with a single substitution at position 338 from arginine to leucine was identified in a patient with juvenile thrombophilia in the Padua University Hospital [43]. Hence this variant referred as FIX Padua displayed an 8-fold higher FIX activity although the plasma levels were normal. Recent studies implementing this hyperfunctional FIX. Padua, also demonstrated its potential to correct the bleeding phenotype by allowing a reduction of the administered lentiviral vector dose for liver targeted expression in mice [44]. In this study the introduction of R338L in canine FIX led to 5-fold higher activity compared to canine wt-FIX. In combination with a codon-usage optimized canine FIX, the FIX Padua mutation allowed an increase of the FIX activity to 125% of the physiological clotting levels resulting in 15-fold gain of gene therapy potency. Long-time correction of the bleeding phenotype was demonstrated in a canine model by expression of the gain-of-function FIX-R338L variant [45]. The transvenular AAV vector mediated delivery of FIX Padua resulted in circulating cFIX levels with a magnitude of expression which was similar to those in earlier observed studies with hemophilia B dogs using wt-cFIX [46]. However the expression of FIX-R338L in the muscles of treated dogs resulted in 8-9-fold higher specific activity and normalization of the whole blood clotting time which was stable throughout the experiment followed up to 5 years [45]. Currently, these encouraging preclinical reports led to the recruitment of clinical gene therapy trial with the FIX Padua based on AAV serotype 8 gene delivery system into the liver (Registration number: NTC01687608). The FIX variants with enhanced specific activity therefore are a powerful tool to overcome the difficulties which are associated with vector delivery. Regarding the wide therapeutic window of FIX, the application of variants with enhanced specific activities may facilitate the success to achieve sufficient clotting activities at lower FIX antigen levels and limited vector doses. On the other hand the reduction of the vector dose and the transgene expression levels would be accompanied by decrease of genotoxicity and immunogenicity and therefore provides a further improvement of the safety profile for gene therapy in hemophilia B.

Engineering FIX variants with enhanced release from the target tissue

Residues 3-11 covering the N-terminal region of the FIX Gla domain are essential for FIX binding to endothelial cells [47]. Studies analyzing binding properties of the Gla domain led to identification of the FIX variants K5A and V10K [48], which exhibited low affinity to collagen IV which is abundant in the extracellular matrix of endothelium and other tissues including skeletal muscle. The clotting activity of the variants, in contrast, is fully preserved. In preclinical studies gene transfer to skeletal muscles using adeno-associated viral (AAV) vectors resulted in improved expression of therapeutic FIX levels in mice and hemophilia B dogs [49,50]. Indeed, in clinical gene therapy trials for severe hemophilia B intramuscular injection of AAV-FIX was proved to be safe, however the obtained FIX antigen levels were below the therapeutic range [51]. As the risk of inhibitor development towards FIX seems to rise with the vector dose and the amount of synthesized FIX per injection site, a high number of injections (>100) would be necessary to acquire therapeutic transgene levels in humans [52,53]. Besides, several reports suggest that FIX expressed in skeletal muscle binds to collagen IV and is therefore retained in the target tissue [49-51]. Concerning these obstacles, mutating the collagen IV binding site in FIX resulted in a 2- to 5- fold increase in antigen levels after gene delivery of the variant FIX K5A / V10K to the muscles in mice in comparison to the wild type protein [42]. The variants with enhanced FIX release from the target tissue could therefore be appropriate for all gene transfer approaches, in which collagen IV limits FIX availability. However, the safety, efficacy and functionality of these variants have to be further elucidated.

Engineering FIX variants to bypass FVIII

Other promising FIX mutations are associated with conformational rearrangements of the 99-loop mimicking the physiologically induced alterations upon cofactor binding and Xase complex formation [54,55]. A FIXa variant with triple mutation (Y259F/K265T/Y345T) exhibited several 1000-fold increases in amidolytic activity toward synthetic peptide substrates and altered substrate specificity [56]. Based on these outcomes [56,57], Milanov et al. explored whether introduction of the mutations Y259F, K265T, and Y345T into the full length FIX protein would give rise to cofactor independent activity under normal physiological conditions by determining its clotting activities in human FIX-, FVIII- and FX-deficient plasma [58]. As a result, the triple variant exposed almost the same FIX clotting activity as wt-FIX and no shift toward FX substrate affinity was observed. Instead this variant displayed an increase in FVIII independent activity compared to wt-FIX. Consistent with previously published studies, demonstrating the single amino acid substitution K265A in FIX was sufficient to increase FVIII independent activity [59], FIX K265T (T) alone was responsible for the FVIII independent activity. Furthermore, by combining K265T with two further mutations (V181I and I383V) in the same study the authors obtained a FIX variant (ITV) with 15.6% activity in absence of FVIII at physiological FIX levels. The variants T and ITV did not exhibit any signs for pre-activation of the FIX zymogen [58]. Using a non-viral gene transfer system [60] expression of the variants T and ITV in FVIII knockout mice resulted in shortening of clotting times, reduced blood loss and recovery of clot formation in the microvasculature as observed by imaging of laser induced vessel injury [58]. Correction of the bleeding phenotype was additionally demonstrated in presence of FVIII inhibitory antibodies [58]. Therefore the usage of FIX variants with independent FVIIII activity might provide an innovative FVIII bypassing therapy for patients with neutralizing anti-FVIII antibodies. The strategy might also serve as gene therapy approach for hemophilia A which could be easily adapted from the current FIX gene transfer approaches which have been developed for hemophilia B [61].

Engineering FIX variants with prolonged half-life for gene transfer

FIX variants with prolonged half-life might offer an additional factor to reduce the vector dose in gene therapy for hemophilia. Genetic fusion of the Fc region from immunoglobulin G to the single chain of FIX resulted in 3-4 fold extended half-life in plasma in

preclinical [62] and clinical trials [63]. Similarly the pharmacokinetic properties of FIX could be also improved by genetic fusion to albumin via cleavable peptides derived from the FIX activation sequence [64]. Half-life increased between 3.4- to 4.7-fold in rats and 3.4- to 4.0-fold in rabbits in comparison to recombinant FIX wild type protein which was confirmed in first clinical trials [64].

FX/FXa

FX is a 488 amino acid polypeptide which displays a similar structural organization as FIX and FVII. During its protein synthesis FX undergoes a serial of post- and co-translational modifications. In this regard, 11 glutamic acid residues within the Gla-domain become γ-carboxylated. In contrast to FIX, the maturation of FX includes an endoproteolytic removal of a tripeptide (Arg140-Lys141-Arg142) and formation of a single disulfide bond between Cys132 and Cys302. The mature two-chain FX zymogen is activated in the blood circulation by the extrinsic (FVIIa/TF) and intrinsic (FIXa/FVIIIa) Xase complex through proteolytic cleavage of the peptide bond Arg194-Ile195. The activated FX (FXa) is so far the unique known physiological activator of prothrombin. During the last years, two major strategies based on either FXa or FX variants were envisaged for bypassing of FVIII- and FIX-deficiencies in hemophiliacs. In the first approach FXa variants with zymogen-like properties are generated which display a higher stability and extended half-life in comparison to wild-type FXa [65] whereas the second approach relies on the generation of zymogenic FX chimera proteins which can be activated by thrombin [66].

Engineering FXa variants with zymogen-like properties

Although direct administration of FXa would be adequate to restore hemostasis in hemophiliacs, the activated protease has an extremely short half-life [67,68] and may possibly trigger uncontrolled activation of the systemic coagulation system [69,70]. Zymogen-like FXa variants were therefore investigated to overcome these limitations. Amino acid substitutions which result in zymogen-like FXa were initially described to impair conversion of FXa into the protease state and to diminish its binding affinity to Na^+ and factor Va [71]. FX is activated by cleavage at bond Arg194-Ile195 resulting in the formation of a new N-terminus which is inserted into a binding pocket of the heavy chain by forming of a salt bridge between Ile195 and Asp378 [72]. This critical event is accompanied by various rearrangements of several surface loops at the catalytic domain which contribute to the maturation of the active state of FXa [73]. Bunce et al. could show that the amino acid substitutions I195L and V196A are crucial for the zymogen-like properties in FXa [65]. Both FXa variants exhibited only a poor activity in absence of FVa and were not affected by inactivation of antithrombin III and tissue factor pathway inhibitor (TFPI). Moreover, association of I195L-FXa and V196A-FXa with FVa on activated platelets was demonstrated to entirely recover their clotting activities. Additionally, FXa-I195L and FXa-V196A showed prolonged half-lives compared to wt-FXa in hemophilic plasma (approximately 1 minute *vs.* about 60 minutes) and provide an efficacious bypassing of the intrinsic pathway. Most recently, Ivanciu et al. demonstrated that FXa-I195L exhibits a 30- to 60-fold extended half-life than *in vivo* and that its infusion does not cause extreme activation of the systemic coagulation [74]. Furthermore, administration of the variant was shown to correct aPTT in a dose dependent manner. In the same study, the investigators also evaluated the hemostatic efficacy of FXa-I195A in comparison to the biopharmaceutical bypassing agent FVIIa [75] using a tail clip and $FeCl_3$-induced injury in carotid artery models. A 10-fold higher dose of administrated hFVIIa dose (3–5 mg/kg) was required to obtain similar efficacy in comparison to human I195A-FXa. Comparable high concentrations of hFVIIa were also necessary to induce vessel occlusion in the $FeCl_3$ injury model in hemophilia B mice. The proposed FXa variants with zymogen-like properties therefore might be an alternative to FVIIa in gene therapy for inhibitor patients. Nevertheless, additional preclinical and clinical studies would be necessary to evaluate their safety profile.

Thrombin-activable zymogenic FX chimera proteins

To generate a thrombin-activable zymogenic FX chimera Louvain-Quintard et al. replaced the activation domain in FX by the corresponding sequence of fibrinogen referred to as fibrinopeptide A (FpA) [66]. The FX hybrid protein can be directly activated by thrombin cleavage and in turn activate prothrombin to thrombin. This shortcut re-establishes a shortened intrinsic amplification loop of thrombin formation in absence of the Xase complex [66]. Since an effective production of fibrin is accompanied by rapidly release of FpA from fibrinogen, the investigators reasoned that chimera FX_{FpA} may display equally high susceptibility to activation by thrombin as its natural substrate fibrinogen. Indeed, incubation of the FX_{FpA} with thrombin resulted in generation of physiological FXa concentrations [76]. FIX_{FpA} were sufficient to correct thrombin generation by enabling loop amplification through reciprocal activation between thrombin/FX_{FpA} and FXa/prothrombin and normalized clotting times in FVIII- and FIX-deficient plasma. Other groups also described self-activating FX variants in which either the activation peptide was replaced by that of prothrombin [77] or auto-activation was initiated by deletions of 41 C-terminal residues in the activation domain of FX [78]. The thrombin-activable zymogenic FX chimera proteins therefore might represent an interesting alternative to bypass FVIII and FIX deficiencies.

General Developments

Bioengineered vitamin K-dependent proteases, factor VII, factor IX and factor X, could provide a possibility to optimize available gene therapy approaches for hemophilia. Protein modification might further provide novel treatment possibilities for patients with inhibitors bypassing the regular coagulation cascade and therefore acting independent of absence or presence of neutralizing anti-FVIII or anti-FIX antibodies. In several preclinical and clinical studies the potential of bioengineered clotting factors to induce effective hemostasis has been already demonstrated. An overview of the literature is given in table 1. Based on the improved features, like enhanced activity, plasma stability, and improved bypassing activity, the antigen levels required to restore hemostasis by gene therapy would be less than using non-

Strategy	FVIIa	FIX	FX/FXa
Increased activity	[4,10,16-22, 24, 35]	[37, 40-42, 44-46]	/
Affected membrane affinity	[5, 25-27]	[42,48]	/
Prolonged half-life	[28-30]	[62-64]	[65,71,74,75]
FVIII bypass activity	/	[56-59]	/
Thrombin- / auto-activable	/	/	[66,77,78]

Table 1: Summary of references in bioengineering strategies of factor VII, IX and X.

engineered wild-type proteins. This could make gene therapy also safer as vector dose and number of injection sites could be reduced. Additionally, novel functions, like the FVIII independent activity of FIX variants, might allow gene therapeutic approaches for patients, such as patients with inhibitory antibodies against FVIII, for which a FVIII gene transfer would not have been considered adequate. Since for many gene transfer vectors, like AAV, transgene size is a limiting factor and FVIII gene packaging might be problematic, replacing FVIII with FVII, FIX, or FX might be a viable option. The thrombogenicity of these bioengineered variants seems to be highly dependent on the expression levels and the specific protein construct. In general, physiological or even lower transgene levels should manage/control bleedings without a risk of thrombotic events, especially considering the natural protective effect due to the phenotype in hemophilia. Overall, immunogenicity of bioengineered variants might be problematic. The development of anti-drug antibodies in the recent phase III NovoNordisk study with modified FVIIa has not been predicted in preclinical or clinical trials. Such a surprising finding therefore puts in question protein modification approaches for diseases like hemophilia in general since the developmental risk involved in novel products is difficult to judge. The development of test systems for better predictability of such events is therefore of such utmost importance.

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