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Commentary

Recent advances in the development of coagulation factors and procoagulants for the treatment of hemophilia

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ABSTRACT

Hemophilia is a family of rare bleeding disorders. The two primary types, hemophilia A and hemophilia B, are caused by recessive X-chromosome linked mutations that result in deficiency of coagulation factor VIII (FVIII) or factor IX (FIX), respectively. Clinically, hemophilia is manifested by spontaneous bleeding, particularly into the joints (haemarthrosis) and soft tissue, and excessive bleeding following trauma or surgery. The total overall number of hemophilia patients worldwide is approximately 400,000, however only about 100,000 of these individuals are treated. The first treatment of hemophilia was initiated when it was determined that the clotting deficiency could be corrected by a plasma fraction taken from normal blood. The discovery of factor VIII enrichment by cryoprecipitation of plasma opened a new era of therapy which eventually led to the production of factor concentrates and the subsequent development of highly purified forms of plasma factors. The most significant improvements have been the availability of recombinant forms of factors VIII and IX. Unfortunately, recombinant factors still retain some of the limitations of plasma concentrates. These limitations include development of antibody responses in patients and the relatively short half-life of the molecules requiring frequent injection to maintain effective concentration. Treatment beyond replacement of native factors has been tried. They include the development of modified factor VIII and IX molecules with improved potency, stability and circulating half-life and enhancement of a prothrombotic responses and/or stabilization of coagulation factors via inhibition of key negative regulatory pathways. These approaches will be reviewed in this commentary.

1. Introduction

Coagulation is the formation of a stable fibrin/cellular hemostatic plug that is sufficient to stop bleeding. The coagulation process involves complex biochemical and cellular interactions that can be divided into four stages (Fig. 1). Stage one is the formation of activated factor X by either the contact (intrinsic) or the tissue factor cellular injury pathway (extrinsic). Stage two is the formation of thrombin from prothrombin by Xa. Stage three is the formation of fibrin from fibrinogen by thrombin mediated proteolysis. Stage four is fibrin stabilization by factor XIIIa cross linking. Hemophilia is defined as a congenital or acquired disorder of coagulation that usually, but not always, involves a quantitative and/or functional deficiency of a single coagulation protein. Hemophilia is a family of rare bleeding disorders. The two primary types, hemophilia A and hemophilia B are caused by recessive X-chromosome linked mutations that result in deficiency of coagulation factor VIII (FVIII) or factor IX (FIX), respectively. Clinically, hemophilia is manifested by spontaneous bleeding, particularly into the joints (haemarthrosis) and soft tissue, and excessive bleeding following trauma or surgery.

The gene for factor VIII was cloned in 1984 [1,2] and maps to Xq28 of the long arm of chromosome X, distal to the glucose 6-phosphate dehydrogenase gene and about one megabase from the Xq telomere [3,4]. Sites of synthesis of factor VIII, based on mRNA localization, have been identified in whole normal liver and isolated hepatocyte (primary site of expression), in spleen and lymph nodes and in lesser amounts in pancreas, kidney, muscle, and placenta [5]. The factor VIII gene has a translated molecular weight of 265 kDa and encodes a 2332 amino acid protein and a 19 amino acid signal peptide [2]. The molecular weight of the mature, secreted proteins can vary depending on the extent of post-translational glycosylation. The protein is composed of distinct domains termed A, B and C. There are three copies of the A domain [A1, A2, A3] with about 30% sequence homology and a 35% sequence homology to the plasma protein ceruloplasmin and factor V [2,6]. Domain A2 is separated from A3 by the B domain. The B domain contains 19 of the potential 25 glycosylation sites for factor VIII [7]. There are two copies of the C domain [C1 and C2] with approximately 40% sequence homology to each other. Factor

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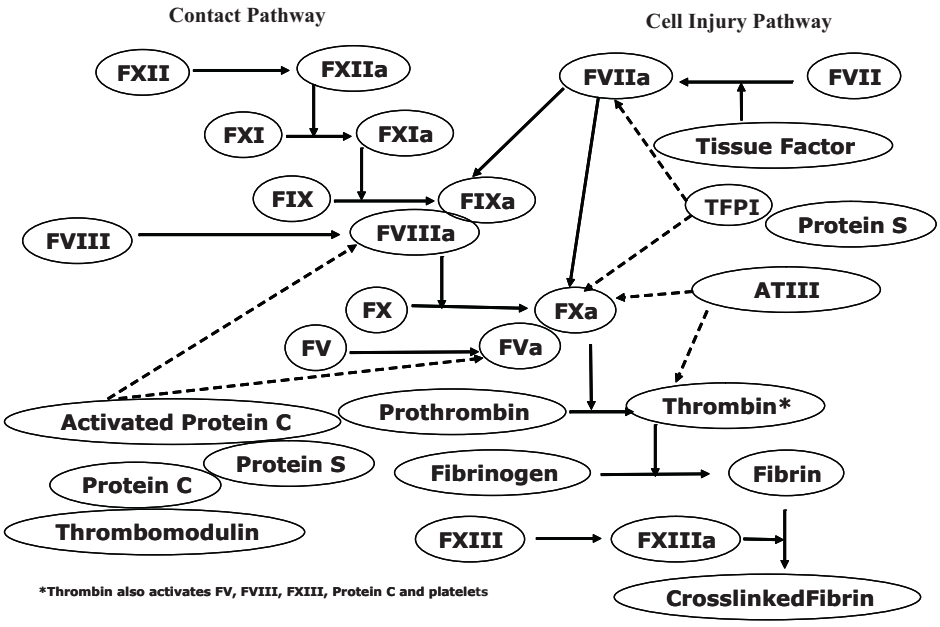

Fig. 1. Coagulation is the formation of a stable fibrin/cellular hemostatic plug that is sufficient to stop bleeding. The coagulation process involves complex biochemical and cellular interactions that can be divided into four stages. Stage 1 is the formation of activated factor X by either the contact (intrinsic) or the tissue factor (extrinsic) pathway. Stage 2 is the formation of thrombin from prothrombin by Xa. Thrombin has a wide range of effects that serve to accelerate coagulation by activating several key co-factors involved in clotting and also by activating key regulatory proteins that modulate and stop the clotting process. Stage 3 is the formation of fibrin from fibrinogen by thrombin mediated proteolysis. Stage 4 is stabilization of the clot by factor XIIIa mediated cross-linking of fibrin.

VIII circulates at a concentration of 100–200 ng/mL in association with von Willebrand factor (vWF). The factor VIII molecule functions by accelerating the proteolytic activation of factor X by factor IXa in a reaction requiring calcium and a phospholipid surface. The reaction is increased in the presence of catalytic amounts of thrombin (Fig. 1) [8]. Factor VIII is inactivated by Activated Protein C and its cofactor Protein S. Inactivation of factor VIII is produced by cleavage primarily at arginine-336 [9,10] (Fig. 1). The factor VIII coagulant activity is assumed to be 100% or one unit of factor VIII activity per mL of normal plasma (~5000 U/mg) [11]. The importance of factor VIII for clotting efficiency is demonstrated by the fact that absent or deficient levels of factor VIII activity result in severe bleeding. Additionally, a correlation between severity of bleeding tendency and low level of circulating factor VIII has been observed. Hemophilia A patients with <1% factor VIII activity have severe, frequent spontaneous bleeding; those with 1–5% activity have moderately severe bleeding and some spontaneous bleeding after minor trauma; those with >%5 activity have mild disease with prolonged bleeding only after significant trauma or surgery [11,12].

The gene for factor IX was cloned in 1982 and maps to Xq27.1-q27.2 on the long arm of chromosome X [13–17]. Factor IX is a 55,000 MW plasma protein member of the serine protease family of coagulation factors that circulates in the plasma at approximately 4 μg/mL (~250 U/mg) [18,19]. FIX is synthesized as a single polypeptide chain that undergoes extensive post-translational modifications including signal peptide cleavage, disulfide bond formation, glycosylation, vitamin K-dependent gamma-carboxylation of glutamic acid residues in the NH_2 terminal region, beta-hydroxylation and propeptide cleavage [19]. The liver appears to be the primary site of factor IX synthesis [18]. Hepatocytes directly secrete factor IX into the plasma and liver disease decreases plasma concentration of factor IX [20]. Factor IX is proteolytically activated to factor IXa by either activated factor VII in the presence of tissue factor, calcium ion and phospholipid, or by factor XIa in the presence of calcium ions [21] (Fig. 1). Factor IXa then activates factor X in the presence of activated factor VIII, phospholipid and calcium ions (Fig. 1). The critical role of factor IX in coagulation is evident in the hemophilia B patients who exhibit bleeding problems that range from hemorrhagic complications after surgery or physical trauma to frequent, unprovoked, spontaneous bleeding episodes in joints and soft tissues. As with factor VIII, factor IX coagulant activity is assumed to be 100% or one unit activity per mL of plasma. The classification of mild, moderate and severe deficiency is similar to that for factor VIII.

The first treatment of hemophilia was initiated when it was determined that the clotting deficiency could be corrected by a plasma fraction taken from normal blood [22]. This factor was subsequently described as anti-hemophilic globulin [23]. A role for factor VIII was suggested by Brinkhous in 1947 [24]. However, the observation that plasma from one hemophiliac could correct the clotting defect in another bleeding patient suggested that more than one coagulation factor might be involved in mediating individual bleeding disorders. Further study resulted in the designation of factor VIII deficiency as hemophilia A and a second deficiency as hemophilia B [25,26]. The deficiency in hemophilia B was subsequently identified as factor IX. The discovery by Pool [27] of factor VIII enrichment by cryoprecipitation of plasma opened a new era of therapy which eventually led to the production of factor concentrates and the subsequent development of highly purified forms. The most significant improvements have been the availability of recombinant forms of factors VIII and IX. These highly purified recombinant molecules have proved to have a safety and efficacy profile that has made them the primary form of replacement factors used in the treatment of hemophilia in the developed world.

Recombinant FVIII and FIX still retain some of the limitations of plasma concentrates. These limitations include development of antibody responses in patients, the short half-life of the molecules that requires frequent intravenous infusions to maintain effective plasma concentration, and the cost of factor replacement. Attempts to improve treatment options for hemophilia include development of modified factor VIII and IX molecules with improved potency, stability and circulating half-life. Alternative

Table 1
Summary of various factor modifications aimed at improving hemophilia treatment.

Factor	Modification	Improvement	Reference
Factor VIII			
Pegylated liposome	Recombinant FVIII reconstituted with the PEGylated liposomes (DSPE PEG2000) (BAY79-4980) binds non-covalently to PEG on the liposome surface with a Kd of 1.9–4.5 nM	Half-life and efficacy were not enhanced with this formulation approach and clinical studies have been terminated. PEGYlated $t_{1/2}$ = ~10 h, FVIII $t_{1/2}$ = ~10 h	[31–33]
B-Domain deleted	ReFacto®, Xyntha®, "N8"	No major biochemical, functional or pharmacokinetic differences with full length FVIII has been noted and these molecules do not have improved efficacy or prolongation of half-life compared to currently available FVIII molecules. Deleted B domain may improve manufacturing and characterization. Non-antibody affinity purification of ReFacto® and Xyntha® Full length FVIII $t_{1/2}$ = ~10 h, ReFacto® $t_{1/2}$ = ~11 h, Xyntha® $t_{1/2}$ = ~11 h, N8 $t_{1/2}$ = ~10 h	[35–37]
Direct PEGylation of FVIII	A site directed mutagenesis was performed to produce 23 FVIII variants with introduced surface exposed cysteine on a B-domain deleted FVIII. The cysteines were conjugated with 5–60 kDa PEG by reaction with PEG-maleimide	Some of these molecules retained FVIII activity in vitro and in vivo, were appropriately processed, maintained affinity to VWF, were protected against inhibition by site directed antibodies from inhibitor patients and showed improved half-lives in FVIII KO mice of about 2-fold (8 h vs. 12–15 h) over rBDDFVIII. The half-lives of a 60 kDa PEG-K1804C and 60 kDa diPEG-L491C/K1804C was extended ~20-fold compared to rBDD-FVIII in VWF knock-out mice, however this difference was due to the rapid clearance of non-PEGylated FVIII because of the absent VWF. The half-lives of the PEGylated molecules were no longer than non-PEGylated FVIII in the presence of normal VWF concentration. This suggests that VWF binding predominates in regulating FVIII half-life. A prolonged hemostatic correction following treatment of FVIII deficient mice was also observed for the 60 kDa diPEGylated FVIII. Mice were subjected to tail transection 48 h after treatment with full-length FVIII or 60 kDa PEGylated FVIII. Eighty-five percent of the PEGylated mice survived vs. 60% survival of the full-length FVIII treated mice	[34]
FVIII-Fc fusion	A single FVIII molecule is fused to the Fc region of human IgG1	This molecule has a similar activity and efficacy to BDD rFVIII, but has a two-fold increased half-life compared to the non-conjugated molecule (~7 h vs. ~15 h). The half-life prolongation occurs because the Fc domain promotes binding to the neonatal Fc receptor (FcRn) which has a critical role in IgG homeostasis by protecting the molecules from degradation and promoting a recycling of the IgG molecules to prolong their circulating half-life	[38,39]
Factor IX			
FIX-Fc fusion	A single FIX molecule is fused to the Fc region of human IgG1	The half-life of rFIXFc is approximately 3–4 fold longer than that of rFIX (~12 h vs. ~46 h) in FIX KO mice, hemophilia A dogs and normal non-human primates	[39,40]
Catalytic domain mutations	Five full-length mutants of FIX with amino acid substitutions in the catalytic domain were evaluated	Two of the mutant molecules (Y94F/K98T/Y177F/I213V/E219G and Y94F/A95aK/K98T/Y177F/I213V/E219G) had significantly increased catalytic activity in model systems. They activated FX with a k_{cat}/K_m that was increased 17 and 6 fold for the two mutants, respectively. However, they were no more effective in plasma assays than plasma derived FIX	[41]
FIX Padua	Natural R338L mutation	A 5–10 fold higher specific activity compared to WT FIX may provide some benefit in the development of gene therapy vectors where low expression could be offset by the higher specific activity of this FIX variant	[42]
Alternate approaches			
Site specific mutated FVIIa	Two FVIIa mutations have been developed. These are NN1731 (V158D/E296V/M298Q-FVIIa) and BAY 86-6150 which has N-linked glycosylation sites added via the mutations T160N and V253N through the addition of two N-glycans by a directed glycosylation reaction and four amino acids engineered in the Gla-domain (P10Q; K32E;A34E;R36E) to increase γ-carboxylation	NN1731 (119 s) demonstrates greater activity compared to rFVIIa (881 s) in shortening clotting time of Hemophilia A plasma (1424 s), increasing clot stability and increasing platelet dependent factor Xa generation. BAY 86-6150 has 3-fold improved thrombin generation time and peak thrombin generation vs. FVIIa and the half-life is increased two fold	[45–48]

Table 1 (*Continued*)

Factor	Modification	Improvement	Reference
PEGylated FVIIa	FVIIa PEGylation used a process that removes terminal sialic acid from the molecule by neuraminidase followed by addition of sialic acid PEG groups of various sizes (5, 10, 20, 40 kDa) using sialic acid transferase	PEGylation increased the half-life that correlated with a prolonged procoagulant response that was maintained for 25 h vs. 4–5 h for nonPEGylated FVIIa	[49]
Site specific Factor Xa mutations	Factor Xa mutations (I161L; V17A) that destabilize the zymogen to protease conversion resulting in a free enzyme with low active binding site and function that is rescued with saturating FVa concentration	These mutations produce a Xa molecule that is primarily active in the localized prothrombinase complex associated with FVa, thus reducing the risk of systemic thrombogenicity by keeping the procoagulant response localized to areas of local injury. This Xa has an improved plasma stability and resistance to protease inhibition. WT-FXa was rapidly inhibited in normal and hemophilia plasma with a half-life of ~1 min while the half-lives of the mutated FXa molecules was ~60 min in normal plasma and ~90 min in hemophilia plasma. This Xa mutant could be a substitute for FVIIa in the treatment of inhibitor patients	[50]
Protein C antagonists	Peptide based APC inhibitors, which mimic residues surrounding the APC cleavage site at Arg306 of FVa, have been synthesized. These peptides are specific and reversible inhibitors of APC [K_i ~ 1–2 μM]	Specific and reversible inhibitors of APC (K_i 1–2 μM). Representatives of this group of compounds inhibit FVa inactivation by APC and prolong FVa functional activity in the prothrombinase complex. In the absence of inhibitor ~90% of FVa activity is lost after 5 min. In the presence of 10-fold K_i 40% of FVa activity remains after 60 min	[54]
Tissue factor pathway inhibitor antagonists	A non-anticoagulant polysaccharide extract from brown algae (AV513) and an aptamer (ARC19499)	TFPI inhibition by AV513 had a positive effect on restoration of a normal coagulation profile and, in the dog hemophilia model, an improved clot formation and a reduction in bleeding time by both IV and oral dosing. Clotting time was reduced from ~25 min in non-treated dogs to ~5 min in AV513 treated (15 mg/kg) dogs. ARC19499 restores the normal coagulation in FVIII or FIX deficient blood/plasma and it corrects coagulation in non-human primates made deficient in FVIII by anti-FVIII antibody depletion. Clotting times increased from ~5–10 min for animals with normal FVIII to ~20–40 min with FVIII depletion. Treatment with ARC19499 corrected clotting time to ~10 min	[60,61]

approaches have attempted to enhance the prothrombotic response and/or stabilize coagulation factors via inhibition of key negative regulatory pathways.

2. Factor VIII

Bleeding episodes in hemophilia patients are treated with intravenous infusions of replacement factor sufficient to stop the bleeding episode. These treatments are termed "on demand" to reflect the immediate nature of these treatments. Intravenous administration at intervals of 2–3 times a week can also be given to protect against bleeding. These treatments are termed prophylactic infusions. The need for 2–3 infusions is due to the short half-life of FVIII of 12–14 h [28]. The majority of efforts to improve FVIII have focused on either increasing the half-life and/or the bioactivity of FVIII in order to reduce the frequency of infusions and/or the amount of factor infusion required to achieve hemostasis. The primary half-life extension strategy involves conjugation of FVIII or use of formulations with high molecular weight molecules such as polyethylene glycol (PEGylation) or hydroxyethyl starch (HESylation). These conjugates can greatly increase the half-life of protein therapeutics [29]. One approach evaluated was the use of a formulation in which the liposome component was PEGylated but not the FVIII molecule [30–33]. Recombinant FVIII reconstituted with the PEGylated liposomes (DSPE PEG2000) binds non-covalently to PEG on the liposome surface with a Kd of 1.9–4.5 nM. [31]. The FVIII does not have altered coagulation activity and binds to its carrier protein, von Willebrand Factor (VWF), with the same affinity [31] (Table 1). It was thought that the liposomes, because of their lipid composition, might enhance the localization of the associated FVIII with the procoagulant platelet membranes and enhance local coagulation response in addition to increasing the plasma half-life of FVIII [31–33]. Unfortunately, neither half-life nor efficacy was enhanced with this formulation approach and clinical studies have been terminated. Direct PEGylation of FVIII is also being assessed using a site-specific PEGylation reaction to avoid random PEGylation that can result in more than one PEG molecule per FVIII [34]. Random PEGylation can result in reduced coagulation activity and difficulty in characterization of the manufactured protein. A site directed mutagenesis was performed to produce 23 FVIII variants with introduced surface exposed cysteine on a B-domain deleted FVIII (rBDD-FVIII). The cysteines were conjugated with 5–60 kDa PEG by reaction with PEG-maleimide. Some of these molecules retained FVIII activity in vitro and in vivo, were appropriately processed, maintained affinity to VWF, were protected against inhibition by site directed antibodies from inhibitor patients and showed improved pharmacokinetics with a 2-fold increase in half-life vs. non-PEGylated FVIII (Table 1). The fact that PEGylation did not result in significantly longer half-life that is typical of PEGylated proteins suggests that clearance of the FVIII carrier protein, VWF, may predominate in FVIII clearance. This was confirmed in VWF knock out (KO) mice in which two of these PEGylated FVIII molecules (60 kDa PEG-K1804C and 60 kDa diPEG-L491C/K1804C) exhibited a half-life extension that was ~20-fold longer compared to rBDD-FVIII. However, this increased half-life was the result of

the rapid clearance of the rBDD-FVIII due to the absence of VWF. The half-life of the PEGylated FVIII molecules was still in the range of rBDD-FVIII half-life in mice with normal VWF levels. This suggests that VWF binding predominates in regulating FVIII half-life. A longer hemostatic correction following treatment of FVIII deficient mice was also observed for the 60 kDa PEGylated FVIII. Mice were subjected to tail transection 48 h after treatment with full-length FVIII or 60 kDa PEGylated FVIII. Eighty-five percent of the PEGylated mice survived vs. 60% survival of the full-length FVIII treated mice suggesting that more of the mice receiving PEGylated FVIII sustained hemostatic levels of FVIII. This observation is consistent with the 2-fold longer half-life of these molecules vs. rBDD-FVIII and suggests that these PEGylated molecules could be expected to provide the patient a reduced number of infusions for prophylactic therapy and perhaps a more effective hemostatic response with on demand therapy due to the longer duration of effective therapeutic concentrations. Structurally modified FVIII molecules such as the approved B-domain deleted ReFacto® and Xyntha® have also been developed [35] (Table 1). The deletion of the B domain does not appear to impact efficacy and pharmacokinetics but may have some benefit in terms of manufacturing, characterization and activation. A newer modified FVIII known as N8 is also being evaluated. This molecule retains 21 residues of the B domain (SFSQNSRHPSQNPPVLKRHQR) as a linker sequence between the A2 domain and the A3 domain (Table 1). This sequence represents 10 amino acids from the N-terminal of the B domain and 11 amino acids from the C-terminal of the B-domain. The maintenance of the C-terminal sequence ensures a FVIII heavy and light chain variant. No major biochemical, functional or pharmacokinetic differences with full length FVIII has been noted and this molecule would not be expected to have an improved efficacy of prolongation of half-life compared to currently available FVIII molecules [36,37]. A Factor VIII-Fc conjugate has also been described (Table 1). A single FVIII molecule is fused to the Fc region of human IgG1. This molecule has a similar activity and efficacy to rBDD-FVIII, but has a two-fold increased half-life compared to the non-conjugated molecule. This longer half-life should result in improved treatment regimens for both on demand and prophylactic treatment [38]. The half-life prolongation occurs because the Fc domain promotes binding to the neonatal Fc receptor (FcRn) which has a critical role in IgG homeostasis by protecting the molecules from degradation and promoting a recycling of the IgG molecules to prolong their circulating half-life [39].

3. Factor IX

Factor IX is a smaller (~55 kDa) and less complex molecule than FVIII (~280 kDa), with approximately a 2-fold longer half-life than FVIII [28,40]. However, similar attempts to improve half-life and bioactivity are being assessed to reduce frequency and dose of factor required for on demand and prophylaxis of hemophilia B patients. The most mature of these modifications is the development of a factor IX monomeric Fc fusion protein (rFIXFc) [39] (Table 1). The half-life of rFIXFc is approximately 3–4 fold longer than that of rFIX and plasma derived FIX. Both FIX deficient mice and Hemophilia B dogs had approximately a 2-fold longer correction of whole blood clotting time compared to rFIX (Table 1). Treatment with rFIXFc would be expected to significantly reduce the intervals required for maintaining prophylactic levels of FIX in the hemophilia B patient. Mutation of FIX to enhance catalytic activity has also been attempted by production of mutants in the catalytic domain [41]. Two of the mutants (Y94F/K98T/Y177F/I213V/E219G and Y94F/A95aK/K98T/Y177F/I213V/E219G) demonstrated significantly increased catalytic activity in model systems (Table 1). They activated FX with a k_{cat}/K_m that was increased 17 and 6 fold for the two mutants, respectively. However, they were no more effective in plasma assays than plasma derived FIX and would, most likely, not result in an improvement in treatment. Recently, a natural mutation of FIX has been reported with a single point mutation (R338L, FIX Padua) that has a 5–10 fold higher specific activity compared to WT FIX [42] (Table 1). The therapeutic utility of FIX Padua is yet to be determined, but it may provide some benefit in the development of gene therapy vectors where low expression could be offset by the higher specific activity of this FIX variant.

4. Alternate approaches

4.1. Factor VIIa

A significant subpopulation of haemophiliacs exhibits inhibitory antibody responses that render such patients refractory to therapy with coagulation factor concentrates. Approximately 10–15% of hemophilia A patients and 1–3% of hemophilia B patients develop persistent antibody inhibitors to factor concentrates [43,44]. Also, up to 50% of hemophilia B patients with inhibitors are at risk for severe allergic reactions, including anaphylaxis, in response to FIX administration [44]. Elimination of persistent inhibitors is possible in some hemophilia A patients by immune tolerance induction therapy. Alternative treatment options for hemophilia A and hemophilia B patients with inhibitors include agents such as recombinant activated Factor VIIa (rFVIIa) and plasma derived prothrombin complex concentrates (PCC) that bypass deficiencies of intrinsic coagulation factors [45] (Table 1). rFVIIa has a very short half-life in adult (2.6 h) and pediatric (1.3 h) patients. This requires frequent doses to achieve control of bleeding and significantly limits the use of rFVIIa in prophylactic treatment. The frequent doses and variable response of individual patients can also result in a risk of thrombosis. The attempts to enhance the activity and pharmacokinetics of FVIIa have used both novel PEGylation strategies and site directed mutagenesis. One of these, a mutant form of rFVIIa (V158D/E296V/M298Q-FVIIa; NN1731) (Table 1) demonstrates greater activity compared to rFVIIa in shortening clotting time, increasing clot stability and increasing platelet dependent factor Xa generation [45–47]. Another rFVIIa was modified to improve pharmacokinetics and potency by specific point mutations (Table 1). The molecule, BAY 86-6150 has N-linked glycosylation sites added by mutating two sites (T160N and V253N) to improve pharmacokinetics through the addition of two N-glycans by a directed glycosylation reaction and four amino acids engineered in the Gla-domain (P10Q; K32E;A34E;R36E) to increase γ-carboxylation and enhance localization at bleeding sites. Thrombin generation time and peak thrombin generation was improved three-fold vs. rFVIIa and half-life was increased two fold [48]. These modifications in rFVIIa would be expected to accelerate the correction of bleeding and perhaps reduce the number of treatments required to produce a hemostatic effect by both enhancing the rate of clot formation and by localizing the stimulation of clotting at the site of bleeding. rFVIIa has also been PEGylated to improve its pharmacokinetics and perhaps expand its therapeutic use as a prophylactic treatment for hemophilia patients (Table 1). FVIIa PEGylation used a process that removes terminal sialic acid from the molecule by neuraminidase followed by addition of sialic acid PEG groups of various sizes (5, 10, 20, 40 kDa) using sialic acid transferase to two sites [49]. This PEGylation resulted in an increased half-life that correlated with a prolonged procoagulant response and perhaps a rFVIIa molecule with better prophylactic properties [49].

4.2. Factor Xa mutants

Factor Xa mutation (I161L; V17A) has resulted in Xa molecules that are most active in the localized prothrombinase complex

(Table 1). This localization would be expected to reduce the risk of systemic thrombogenicity by keeping the procoagulant response localized to areas of local injury where hemostasis is required. Non-specific systemic activation of the clotting cascade would be significantly limited. In addition, these changes have improved the plasma stability and resistance to protease inhibition of these mutants. FXa is rapidly inhibited when incubated in normal and hemophilia plasma with a half-life of ~1 min. The half-lives of the mutated FXa molecules are ~60 min when incubated in normal plasma and ~90 min in hemophilia plasma. A Xa molecule with these protease resistance characteristics combined with some modification to enhance in vivo circulating half-life could provide a new generation bypass agent with less thrombogenic risk for the treatment of hemophilia patients with inhibitory antibodies to either FVIII or FIX [50].

5. Control of negative regulatory pathways

An additional approach to the treatment of hemophilia is through enhancement of a prothrombotic response and/or stabilization of coagulation factors via inhibition of key negative regulatory pathways. Protein C and Tissue Factor Pathway Inhibitor are two targets that have a profile suggesting a strategy of inhibiting these pathway inhibitors could provide a novel single treatment or an adjuvant treatment given in addition to recombinant purified factors (Table 1).

5.1. Inhibition of Activated Protein C [APC]

Protein C is a vitamin K-dependent serine proteinase that circulates as a zymogen. Activated Protein C [APC] possesses both anticoagulant and profibrinolytic activity [51]. Activation of the zymogen is achieved by the cleavage of a 1.5 kDa peptide from the N-terminal end of the heavy chain. Although thrombin can directly activate protein C, activation in vivo occurs at an accelerated rate when Protein C is bound to its endothelial cell receptor, EPCR, and thrombin is bound to endothelial cell-associated thrombomodulin. APC can, in turn, inactivate Factors VIIIa and Va and thus antagonize the Xase and prothrombinase complexes. APC has also been shown to promote the fibrinolytic response by antagonizing PAI-1 inhibitor and to have anti-inflammatory/cytoprotective activity [51]. A treatment that inhibits the activation of Protein C, inhibits the protease activity of APC, or impairs the inactivation of Factors Va and VIIIa could be expected to prolong thrombin generation via the Xase complex. The inhibition of APC might also serve to stabilize clots that form by inhibition of lysis. However, any impact of APC inhibition on its anti-inflammatory/cytoprotective role could be detrimental to inhibition of the protein C pathway.

The potential therapeutic benefit of inhibiting APC in the treatment of hemophilia was recently addressed in a review article by van Dijk and colleagues. They assessed the impact of prothrombotic factors on the clinical phenotype of severe hemophilia [52]. Their review of over 9000 papers published between 1963 and 2003 found 7 relevant papers reporting that the APC resistant Factor V Leiden (7/7 papers) was most consistently associated with reduced clinical severity in the hemophilia population. Factor V Leiden is a mutation of FV (R506Q) that reduces APC proteolytic inactivation and promotes a prothrombotic phenotype. Consistent with the findings of van Dijk et al., Schlachterman et al. recently reported that homozygous and heterozygous factor V Leiden hemophilia A and B mice demonstrated improved hemostasis compared to mice with WT Factor V [53]. In this study, they found that clot formation induced by laser injury could be normalized in FVIII or FIX KO mice crossed to Factor V Leiden expressing mice. The beneficial effect on hemostasis was observed even when the Factor V Leiden was heterozygous. This would suggest that total inhibition of APC might not be required. This could be an important consideration in regard to concerns about the impact of APC inhibition on its important cytoprotective and anti-inflammatory activity. Peptide based APC inhibitors, which mimic residues surrounding the APC cleavage site at Arg306 of FVa, have been synthesized. These peptides are specific and reversible inhibitors of APC [$K_i \sim 1$–2 μM]. Representatives of this group of compounds inhibit FVa inactivation by APC and prolong FVa functional activity in the prothrombinase complex. One inhibitor partially compensated for the absence of FVIII suggesting that these synthetic APC inhibitors may be useful as adjuvants for hemophilia treatment [54] (Table 1). These APC inhibitors have not entered into clinical studies. This may be related to the relatively high K_i of these compounds and the poor pharmacokinetics associated with peptides. In addition, it is likely that more data needs to be developed to demonstrate that the FV and FVIII proteolytic activity can be inhibited without a negative impact on the important anti-inflammatory and cytoprotective role of APC.

5.2. Inhibition of Tissue Factor Pathway Inhibitor [TFPI]

A second approach to promote a prothrombotic state could be via the up regulation of the tissue factor mediated extrinsic pathway of coagulation. This pathway provides for rapid formation of low levels of thrombin that can serve as the initial hemostatic response and can then participate in the acceleration of the Factor VIII, V and IX dependent intrinsic pathway. Tissue Factor, Factor VIIa, and Xa have a central role in this pathway and they are closely regulated by an endothelial cell associated Kunitz Type proteinase inhibitor, Tissue Factor Pathway Inhibitor [TFPI] [55–57]. Liu et al. [58] suggested that inhibition of TFPI might improve coagulation in the hemophilia patient. Liu describes studies that have demonstrated TFPI deficiency in mice can increase thrombus formation, that TFPI antibodies improved bleeding time in FVIII deficient rabbits and shortened clotting in plasma from hemophilia patients. In the rabbit study, Erhardtsen et al. [59] induced a transient hemophilia A by treating rabbits with a FVIII antibody. This was followed by treatment with either Factor VIII replacement or an antibody specific to rabbit TFPI. The anti-TFPI treatment produced a reduction in bleeding and a correction of coagulation that was similar to that observed with FVIII replacement. The Liu paper reported on the effects of a non-anticoagulant polysaccharide that inhibits TFPI isolated from brown algae. A subsequent paper also assessed this polysaccharide in hemophilia A dogs [60]. In both studies it was found that TFPI inhibition had a positive effect on restoration of a normal coagulation profile and, in the dog model, an improvement in hemostatic profile, including an improved clot formation and a reduction in bleeding time (Table 1). Recently, an aptamer inhibitor of TFPI (ARC19499) has been reported [61]. Aptamers are nucleic acid macromolecules that bind tightly to a specific molecular target. Like all nucleic acids, a particular aptamer may be described by a linear sequence of nucleotides (A, U, T, C and G), that is typically 15–40 nucleotides long [62]. In solution, the chain of nucleotides forms intramolecular interactions that fold the molecule into a complex three-dimensional shape. The shape of the aptamer allows it to bind tightly against the surface of its target molecule. ARC19499, like the other TFPI inhibitors that have been reported, restores the normal coagulation profile in FVIII or FIX deficient blood and plasma and corrects coagulation in non-human primates made deficient in FVIII by anti-FVIII antibody administration. Clotting times increased from ~5–10 min for animals with normal FVIII levels to ~20–40 min with FVIII depletion. Treatment with ARC19499 corrected clotting time to ~10 min [61]. ARC19499 has entered into Phase I clinical trials. This will be the first clinical assessment of treating

hemophilia with an approach that does not involve use of replacement factor, but uses the inhibition of a primary anti-coagulant regulatory pathway to promote a procoagulant response to control bleeding. It will be interesting to follow the outcome of this pioneering study.

6. Conclusions

The past 50 years have seen significant improvement in the treatment of hemophilia. The availability of recombinant Factor VIII, Factor IX and Factor VIIa and improvements in the purification and viral removal of plasma derived factors assures access to safe and effective treatment. However, these current therapies still have limitations related to the need for intravenous administration and frequent dosing to maintain therapeutic levels of clotting factor. The last decade has seen a significant effort to provide improved clotting factors with better pharmacokinetic profiles and enhanced bioactivity. Several of these improved replacement factors are currently in clinical trials. In addition, novel approaches directed at promoting hemostasis by inhibiting the key regulatory pathways are currently being evaluated in preclinical and clinical studies. These new approaches could provide significant improvement in the treatment of bleeding disorders.

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