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REVIEW ARTICLE

The promise and challenges of bioengineered recombinant clotting factors

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Summary. The past 10 years of clinical experience have demonstrated the safety and efficacy of recombinant clotting factors. With the adoption of prophylactic strategies, there has been considerable progress in avoiding the complications of hemophilia. Now, insights from our understanding of clotting factor structure and function, mechanisms of hemophilia and inhibitors, gene therapy advances and a worldwide demand for clotting factor concentrates leave us on the brink of embracing targeted bioengineering strategies to further improve hemophilia therapeutics. The ability to bioengineer recombinant clotting factors with improved function holds promise to overcome some of the limitations in current treatment, the high costs of therapy and increase availability to a broader world hemophilia population. Most research has been directed at overcoming the inherent limitations of rFVIII expression and the inhibitor response. This includes techniques to improve rFVIII biosynthesis and secretion, functional activity, half-life and antigenicity/immunogenicity. Some of these proteins have already reached commercialization and have been utilized in gene therapy strategies, while others are being evaluated in pre-clinical studies. These novel proteins partnered with advances in gene transfer vector design and delivery may ultimately achieve persistent expression of FVIII leading to an effective long-term treatment strategy for hemophilia A. In addition, these novel FVIII proteins could be partnered with new advances in alternative recombinant protein production in transgenic animals yielding an affordable, more abundant supply of rFVIII. Novel rFIX proteins are being considered for gene therapy strategies whereas novel rVIIa proteins are being evaluated to improve the potency and extend their plasma half-life. This review will summarize the status of current recombinant clotting factors and the development and challenges of recombinant clotting factors bioengineered for improved function.

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Introduction

There were several issues that fueled the development of recombinant clotting factors including the development and refinement of molecular biology techniques, particularly the expression of proteins in mammalian cells, the need to increase the capacity of clotting factor concentrates and concerns regarding the infectious disease risk of plasma-derived concentrates in the late 1970s and early 1980s [1]. The gene encoding for factor VIII (FVIII) was sequenced in 1984 [2,3] and was at the time the largest gene ever sequenced. Remarkably, the first patient with hemophilia was infused with recombinant FVIII (rFVIII) in March 1987[4] which was available for clinical use approximately 5 years later. Recombinant factor IX (rFIX) and recombinant factor VIIa (rVIIa) followed several years later giving us a full complement of recombinant protein therapeutics to treat patients with hemophilia A, B and those with inhibitors.

The past 10 years of clinical experience have demonstrated the safety and efficacy of recombinant clotting factors. With the adoption of prophylactic strategies, there has been considerable progress in avoiding the complications of hemophilia. Now, insights from our understanding of clotting factor structure and function, mechanisms of hemophilia and inhibitors, gene therapy advances and a worldwide demand for clotting factor concentrates leave us on the brink of embracing targeted bioengineering strategies to further improve hemophilia therapeutics.

The ability to bioengineer recombinant clotting factors with improved function holds promise to overcome some of the limitations in current treatment, the high costs of therapy and increase availability to a broader world hemophilic population. Most research has been directed at overcoming the inherent limitations of rFVIII expression and the inhibitor response. This includes techniques to improve rFVIII biosynthesis and secretion, functional activity, half-life and antigenicity/immunogenicity. Some of these proteins have already reached commercialization and have been utilized in gene therapy strategies, while others are being evaluated in preclinical studies.

These novel proteins partnered with advances in gene transfer vector design and delivery may ultimately achieve persistent expression of FVIII leading to an effective long-term treatment strategy for hemophilia A. In addition, these novel FVIII proteins could be partnered with new advances in alternative recombinant protein production in transgenic animals yielding an affordable, more abundant supply of rFVIII. Novel rFIX proteins are being considered for gene therapy strategies whereas novel rVIIa proteins are being evaluated to improve the potency and extend their plasma half-life. This review will summarize the status of current recombinant clotting factors and the development and challenges of recombinant clotting factors bioengineered for improved function.

Current status of recombinant clotting factors

rFVIII and rFIX

Preclinical development If one was to ask 'Will bioengineered clotting factors ever make it to the clinic?' the response could be 'They already have and more are coming!' Regardless of how recombinant clotting factors have been embraced as a therapeutic substitute to plasma-derived concentrates for replacement therapy, they should still be considered bioengineered proteins. rFVIII is derived through heterologous transfection of rFVIII DNA plasmids into a nonhuman mammalian cell line, either Chinese hamster ovary (CHO) or baby hamster kidney (BHK) cells. The expressed proteins are secreted into a culture medium that in some cases contains human and bovine protein for stabilization. The recombinant proteins are then purified via various chromatographic techniques. It is remarkable and a testament to the innovation of the biotechnology industry that rFVIII and rFIX share such similarity in their biochemical properties to their plasma-derived counterparts. The CHO and BHK cell lines have demonstrated their capacity to synthesize recombinant clotting factors that include all of the complex posttranslational modifications necessary for their function. For FVIII, these include endoplasmic reticulum (ER) modifications [signal peptide cleavage, disulfide bond formation, the addition of asparagine (N)-linked oligosaccharides and metal ion binding] and further modifications within the Golgi apparatus (complex modification of N-linked oligosaccharides, O-glycosylation, tyrosine sulfation and heavy and light chain cleavage).

Clinical studies: bioequivalence and differences The biochemical characterization of the recombinant clotting factor concentrates has also shown remarkable similarities to plasma-derived concentrates such that the current activity assays for determining concentrate potency and plasma FVIII and FIX levels have remained useful surrogates for guiding the management of patients. In the clinic, the recombinant clotting factors have proven to be highly efficacious and safe whether used in on-demand therapy, prophylactic regimens or surgery. The advantages of increased supply and reduced exposure to human or animal proteins have led to major changes in hemophilia management such that the majority of pediatric patients in the developed world now receive recombinant clotting factors and some countries have adopted them for all their hemophilic patients. In turn, further modifications in the processing and final formulation of recombinant clotting factors have enabled the development of recombinant clotting factors devoid of any exposure to added human or animal proteins [5] essentially closing the loop to any further risk of pathogen contamination.

Despite this success in biotechnology, there remain differences between the recombinant clotting factors and the plasma-derived concentrates. For four decades, the one-stage [activated partial thromboplastin time (APTT-based)] and two-stage (most recently by chromogenic method) activity assays have been used to measure FVIII activity with little discrepancy noted when comparing human plasmas or various FVIII-containing concentrates. However, rFVIII shows a reduced potency in the one-stage assay compared with the two-stage assay when plasma FVIII is used as the standard [6]. A similar discrepancy is observed following infusion of rFVIII into hemophilic patients. Although rFIX is structurally and functionally similar to plasma-derived FIX, there are differences in the posttranslational sulfation and phosphorylation of rFIX that have been associated with a lower *in vivo* recovery after infusion into hemophilia B patients [7,8]. This necessitates a modification of dosing practice for patients. However, with modified dosing the clinical experience is that the bleeding responses to rFIX for on-demand or prophylactic treatment regimens are similar to those reported for plasma-derived FIX [9–11]. That rFIX has been adopted into clinical practice so readily, particularly for pediatric patients demonstrates that treaters and patients are willing to accept such clinical differences because of altered biochemistry if the perceived benefits outweigh the risks or inconveniences.

Immunogenicity The development of neutralizing antibodies to FVIII is one of the most serious complications of hemophilia A therapy as it results in the abrupt attenuation of responsiveness to FVIII replacement. They are primarily encountered in previously untreated patients with severe FVIII deficiency and ≤50 cumulative exposure days to the infused FVIII. The results from several studies of rFVIII in previously untreated patients show a cumulative incidence of 25–32% after a median of 9–12 exposure days[12]. Some of the patient-related factors that influence the formation of these inhibitors include the type of FVIII gene mutation, immune response genes within the major histocompatibility complex and therapy-related factors such as intense exposure to factor during surgery [13,14]. Neoantigenicity of the infused FVIII product may also be a therapy-related risk factor, particularly in previously treated patients. Clusters of inhibitors have been reported in patients switched to plasma-derived FVIII virally inactivated by pasteurization in conjunction with either prior controlled-pore silica adsorption [15] or solvent-detergent treatment [16]. These processes may have modified the

tertiary structure of the FVIII molecule to expose neoantigens. There has also been concern that modification of FVIII, for bioengineering purposes, may also lead to neoantigenicity. This has not been realized with the currently available recombinant clotting factors although debate may continue to rage in this regard. Studies of previously untreated patients with hemophilia A treated with one rFVIII showed that the rate of inhibitor formation was comparable to results observed in studies of previously untreated patients receiving plasma-derived FVIII [17]. Half of the inhibitors observed were transient and low-level and approximately three-quarters of the patients were able to continue successful treatment with rFVIII, with or without immune tolerance therapy. In Canada, following conversion of the majority of hemophilia A patients to rFVIII from plasma-derived FVIII, an analysis by the Canadian Blood Agency concluded that the switch to rFVIII was not associated with an increase in FVIII inhibitor development [18]. Similar studies on rFIX have not demonstrated any increased risk of inhibitor formation [11]. Thus, at least for bioengineered clotting factors that structurally mimic their plasma-derived counterpart, neoantigenicity has not been a problem.

B domain deleted rFVIII Early on in the study of rFVIII expression it was demonstrated that the B domain of FVIII, the equivalent of approximately 38% of the primary cDNA sequence, could be removed without loss of FVIII procoagulant activity [19]. This significantly improved the yield of rFVIII. The increased expression resulted from markedly increased levels of mRNA and increased translation. The reduced size of the B domain-deleted (BDD)-FVIII cDNA also facilitated packaging within certain viral vectors. As BDD-FVIII has a biochemical profile similar to wild-type FVIII there was enthusiasm to adopt it for gene therapy strategies. B domain deleted rFVIII (BDD-rFVIII, ReFacto, Wyeth) remains the first and only modified human rFVIII molecule to come to commercial production [20]. With the B domain deletion, the protein was less prone to proteolytic degradation. Therefore, no addition of plasma-derived albumin was needed for stabilization of the final product [21]. Compared with full-length FVIII, BDD-rFVIII had comparable biochemical properties as a cofactor in the coagulation cascade [20] yet some biological differences remain. Unactivated BDD-rFVIII binds to activated platelets with higher affinity than native FVIII, with thrombin activation further increasing binding affinity [22]. Moreover, FVIII activity assay discrepancies exist in which one-stage clotting assays of BDD-rFVIII activity, using commercial APTT reagents, are consistently about 50% lower than that measured by the two-stage chromogenic assay [23,24]. This assay discrepancy occurs *in vitro* as well as *ex vivo* after plasma analysis from treated patients. BDD-rFVIII also has a higher specific activity (approximately 15 000 U mg^{-1} protein) as measured by the chromogenic assay compared with full-length FVIII (approximately 4000 U mg^{-1}) [20]. The mechanism for this is not determined, however, several studies indicate that BDD-rFVIII has increased sensitivity to thrombin cleavage [25–27].

Clinical studies have shown that BDD-rFVIII provided safe, well-tolerated and effective treatment of hemophilia A [28,29]. Most significantly, rates of inhibitor formation in previously untreated patients with hemophilia A were similar to that observed with full-length rFVIII concentrates demonstrating that, despite such a major modification of the FVIII protein, this bioengineered form of FVIII was not more immunogenic.

The next generation of bioengineered recombinant clotting factors will likely have significant biochemical differences. Some of the modifications in development will require alternative assays to determine potency and dosing and current therapeutic surrogates such as plasma FVIII levels may not reflect their clinical efficacy. Thus, in order to embrace proteins with these biochemical differences for clinical use, the improvements to the overall therapy for the patient (cost, availability, potency, frequency of dosing, inhibitor risk) must be even more significant.

rVIIa

rVIIa (Novoseven, Novo Nordisk) was first approved in Europe in 1996 for the management of bleeding in hemophilic patients with inhibitors. It has proved to be a safe and effective therapeutic although many therapeutic questions remain unanswered [30]. The minimum effective dose required to maintain hemostasis is unknown. Although the recommended dosing for rFVIIa is 90–120 mcg kg^{-1} every 2 h recent clinical studies have suggested that higher initial doses (180–300 mcg kg^{-1}) may result in a more rapid onset of hemostasis [31,32]. It has a short plasma half-life requiring a short interval (~2 h) for follow-up dosing [33]. In addition, the time from onset of the bleed to the initiation of treatment may have a significant impact on efficacy [34]. There is also a lack of laboratory tests of hemostasis that correlate with clinical outcomes. Although dosing of rVIIa solely based on body-weight without assay monitoring could have become a hindrance to its utility in the clinic that has not been the case. Prior to rVIIa, treatment of bleeding episodes in patients with inhibitors relied on activated prothrombin complex concentrates (APCCs) that share a similar clinical conundrum to rVIIa with regards to effective dosing and the lack of appropriate laboratory monitoring. Whereas they both share risks for rare thrombotic complications, APCCs exhibit an anamnestic inhibitor response not seen with the use of rVIIa [35]. Therefore, rVIIa has enough clinical advantages to overcome some of its unique biochemical properties.

The development of novel bioengineered recombinant clotting factors

Novel rFVIII variants

Just as insights from advances in the basic science of recombinant protein expression led to the development of the

first recombinant clotting factors, recent insights into clotting factor structure and function have allowed investigators to engineer additional targeted modifications to generate clotting factors with improved functional properties. The steps toward engineering these novel variants have begun with an understanding of the limitations in recombinant clotting factor properties as they impact on their production and therapeutic efficacy. In turn, key functional regions were mapped and characterized and subsequently, targeted bioengineering strategies could be applied to alter their properties. The availability of animal models to test all of the recombinant clotting factors within their appropriate therapeutic context has allowed rigorous preclinical testing such that the scope of the bioengineered modifications is now limited only by the imagination of the investigators.

Tables 1 and 2 outline the bioengineering strategies currently being examined for FVIII and FIX respectively, highlighting the targeted functional improvements, those novel recombinants in preclinical development and their potential clinical application. The basic science and preclinical testing for many of these have been extensively reviewed previously [36–40].

For FVIII, strategies to improve the efficiency of expression have included modifications that increase mRNA expression (B domain deletion [19,25] or introduction of a truncated FIX intron 1 [41]), reduce interactions with ER chaperones (Phe309Ser mutation within the FVIII A1 domain [42]) or improve the efficiency of ER-Golgi transport (inclusion of a short B domain segment [43]). As these targeted modifications involve different steps within the secretion pathway, they can be combined in the same molecule providing an additive effect in producing substantial increases in the yield of FVIII in heterologous expression systems [43]. Such modifications could contribute to improved efficacy of gene therapy strategies. Most gene therapy applications for hemophilia A have relied on BDD-FVIII because of its advantages of reduced overall size of the cDNA for packaging into viral vectors and increased mRNA levels. However, *in vivo* results have remained poor with plasma FVIII levels typically undetectable ($< 1\%$) or in the 1–4% range in a few patients transiently [44,45]. Thus a FVIII variant with minimal B domain could provide a significant advantage over BDD-FVIII utilizing currently available gene transfer strategies. Incorporation of these modifications into the previous vectors used in the clinic may increase expression 10-fold, into the therapeutic range. In addition, improved expression may allow a reduction in the vector dosing within gene therapy protocols which could reduce undesirable side-effects.

Novel rFVIII variants may improve the efficiency of rFVIII expression in the milk of transgenic livestock. The milk of transgenic livestock can yield an abundant source of complex

Table 1 Bioengineering strategies for FVIII

Targeted functional improvement	Bioengineering strategy	Potential clinical application
Biosynthesis and secretion		
Increased mRNA expression	B domain deletion	Improved efficacy of gene therapy
	Substitution with FIX intron 1	Improved efficiency of commercial production
Reduced ER chaperone interactions	Phe309Ser	Improved efficiency of expression In transgenic livestock
Increased ER-Golgi transport	Sheet B domain containing variants	
Improved functional activity		
Increased activation	Des-(868–1562)FVIII-HCII	Reduced dosage requirements
Resistance to inactivation	Inactivation-resistant FVIII (IR8) (Arg336Ile/Arg562Lys/Arg740Ala/des-794–1689)	Prolonged cofactor activity at subtherapeutic plasma levels
	A2-A3 disulfide-bridged FVIII (Cys664-Cys1826)	Improved efficacy of gene therapy
Improved plasma half-life	LRP-binding site mutations (A2 domain residues 484–509, C2 domain)	Improved efficacy of gene therapy
	Heparan sulfate proteoglycans-binding site mutations (A2 domain residues 558–565)	Prolonged plasma half-life
Reduced Antigenicity	Recombinant porcine BDD-FVIII	Alternative replacement therapy for inhibitor patients
Reduced immunogenicity	Porcine-human hybrids	Reduced inhibitor risk

Table 2 Bioengineering strategies for FIX

Targeted functional improvement	Bioengineerlng strategy	Potential clinical application
Biosynthesis and secretion		
Increased mRNA expression	ATG triplets at translation initiation	Improved efficacy of gene therapy
	Truncated FIX intron 1	Improved efficiency of commercial production
Reduced collagen IV binding	Lys5Ala, Val10Lys	Improved efficiency of expression in transgenic livestock
Improved functional activity		
Increased specific activity	Arg338Ala	Reduced dosage requirements
	FIX-FX hybrids	Improved efficacy of gene therapy

therapeutic proteins such as recombinant hemophilic factors [46]. Only the pig mammary gland has been shown to carry out all of the posttranslational modifications necessary to generate the biological activity and long circulation half-life needed for complex glycoproteins. The cell density of the pig mammary gland is two to three orders of magnitude greater than can be achieved in current mammalian cell culture production technology. Velander and colleagues have demonstrated that human rFIX can be produced at levels up to 100 U mL^{-1} [47]. However, yields of FVIII have been much lower and subject to chain dissociation because of the harsh chelating environment and/or the absence of von Willebrand's factor (VWF) [48]. rFVIII bioengineered for improved secretion efficiency may increase the yield sufficiently in this system to consider this as an alternative for commercial production. The partnering of these strategies would substantially lower the costs and increase the availability of rFVIII to developing countries. In turn, the large amounts of recombinant protein that can be produced from pig milk can make feasible a number of alternative delivery methods including possibly oral. The risk of pathogen transmission with recombinant protein production in transgenic animals can be reduced through maintenance of pathogen-free herds with multiple barriers in the purification process (chromatography, solvent/detergent treatment, and nanofiltration). In addition, swine have been recently shown to be resistant to nutritional challenges of highly infective tissue homogenates from bovine spongiform encephalopathy-infected cattle and can be fed plant-derived diets [46].

FVIII variants, such as IR8 [49] and disulfide-bridged FVIII [50], with increased potency and resistance to inactivation could also improve the efficacy of gene therapy strategies by providing effective hemostasis at lower levels of protein expression. This is the same principle that may give them an advantage as infusates in standard replacement therapy by reducing the protein dose required. Even more exciting is the prospect that molecules resistant to inactivation could have prolonged cofactor activity at subtherapeutic plasma levels. This could extend the half-life of functional hemostasis and perhaps reduce the frequency of prophylactic infusions.

Increasing the plasma half-life of FVIII by interfering with clearance receptors is the subject of ongoing investigation and would have application within gene therapy protocols and as infusates by reducing the frequency of prophylactic infusions. However, basic studies have suggested that FVIII may interact with the low-density lipoprotein receptor-related protein (LRP) [51–53] as well as heparan sulfate proteoglycans (HSPGs) [54]. Nevertheless, when LRP binding is blocked by its classical antagonist, the 39-kDa receptor-associated protein, RAP, FVIII half-life *in vivo* in murine models was significantly prolonged [52,55], providing enthusiasm for pursuing this strategy. Putative LRP binding sites within FVIII have been localized to A2 domain residues 484–509 [52] and the C2 domain [51,56], whereas the putative HSPG binding site has been localized to A2 domain residues 558–565 [54]. Thus single point mutations or even several targeted mutations within a single site are unlikely to significantly alter FVIII clearance by this mechanism. It is likely that multiple targeted mutations in each of the putative binding sites will be required. As these sites lie adjacent to or even overlap key functional domains for interaction with FIXa and VWF, the challenge will be to bioengineer mutations that can reduce interaction with these clearance receptors while preserving the intermolecular interactions of FVIII. In addition, recent studies have suggested that RAP-sensitive determinants other than LRP may contribute to the regulation of plasma FVIII *in vivo* [57]. Thus there may be additional targets to investigate that are important for FVIII clearance.

With inhibitor antibodies developing in approximately 25% of hemophilia A patients in response to replacement therapy, the availability of alternative hemostatic agents remains critical. A recombinant porcine BDD-FVIII would avoid some of the problems associated with commercial plasma-derived porcine FVIII (allergic reactions associated with contaminating porcine plasma proteins, decreased platelet count associated with porcine VWF in the concentrate and contaminating pathogens such as parvovirus) [58]. In addition, porcine-human FVIII hybrids are being investigated to test if they are less immunogenic than current rFVIII preparations. The immunogenicity of a purified human recombinant BDD-FVIII molecule containing mutations at R484A/R489A/P492A was studied in hemophilia A mice [59]. These residues lie within a key 25 residue epitope within the FVIII A2 domain that is frequently the target of anti-A2 domain antibodies. Inhibitory antibody titers in mice receiving the R484A/R489A/P492A mutant were significantly lower than in mice receiving control human BDD-FVIII. These types of FVIII variants would be a significant advantage if they also reduced the incidence of inhibitors in previously untreated hemophilia A patients.

Novel rFIX variants

As rFIX expression has been markedly more efficient compared with rFVIII, there has been less drive to develop novel rFIX variants. Indeed, the preclinical studies of gene therapy strategies for hemophilia B in animal models showed long-term expression of FIX at levels that would be therapeutic in humans (reviewed in [60]). This generated much enthusiasm for the clinical trials of safety and efficacy in humans. Those studies demonstrated that, despite evidence of gene transfer and expression, the vector doses were too low to yield circulating FIX within the therapeutic range [60]. Escalating the vector dose would have been either impractical or increased the risk of vector-related side-effects. Novel FIX variants bioengineered for more efficient mRNA expression [61,62] or for higher potency [63,64] could prove useful even with existing hemophilia B gene therapy strategies by improving efficacy without having to increase the vector dose [65]. If muscle is to continue to be utilized as a target organ for expression of FIX in gene therapy protocols, a novel FIX variant (Lys5Ala, Val10Lys) with reduced affinity for collagen IV (expressed richly in

skeletal muscle) could improve FIX access to the plasma improving the efficacy for this strategy.

Novel rVIIa variants

The rVIIa has several proposed mechanisms for therapeutic efficacy [30]. Its procoagulant activity is proposed to be through both tissue factor (TF)-dependent activity and TF-independent activity (where rVIIa binds directly to activated platelets and monocytes). Through activation of thrombin activatable fibrinolysis inhibitor (TAFI), rVIIa could also exhibit an antifibrinolytic effect. Insights from the structure and function of its protease domain led to targeted mutations to increase its potency. One combination of targeted missense mutations (V158D/E296V/M298Q/K337A-rVIIa) exhibited 50-fold increased proteolytic activity on human factor X [66]. This protein also demonstrated significant enhancement of TAFI-dependent antifibrinolytic potential. Within an antibody-induced murine hemophilia A model, this protein had a three- to fourfold increased potency over rVIIa as measured by shortened bleeding time and reduced blood loss in a tail bleeding assay [67]. Maxygen (Redwood, CA, USA) recently announced the advancement of several novel rVIIa molecules into preclinical development reporting studies in animal models showing enhanced efficacy and an improved *in vivo* half-life. By stimulating more rapid and higher thrombin generation these novel rVIIa variants could have significant advantages over the currently marketed rVIIa.

The challenges of bioengineered recombinant clotting factors

Over the last 40 years we have seen the treatment of hemophilia move from whole plasma to cryoprecipitate to high purity products and ultimately rFVIII technology. One author described the production of FVIII by recombinant technology as 'the biotechnological equivalent of landing men on the moon' [68]. The moon landing in 1969 may have seemed like the penultimate accomplishment, yet arguably current space missions, although struggling to capture only passing interest within the media, are likely to be more important to the advancement of science. Similarly, we have had more than 10 years to accommodate to the recombinant clotting factor era, yet some of the promises of recombinant technology have yet to be realized: affordability, universal worldwide access and even a cure via gene transfer. This is where the bioengineering of clotting factors generates the most interest. Although, the basic science and targeted engineering strategies have generated enthusiasm for novel recombinant variants, challenges remain that limit their preclinical development.

Scale up for preclinical testing

Heterologous expression in mammalian cell cultures is still the mainstay of bioproduction of rFVIII and rFIX. This remains a relatively inefficient methodology, particularly for rFVIII expression, limiting the quantities available for preclinical studies. Many laboratories have been able to purify quantities sufficient for detailed biochemical analysis and small animal studies. However, pharmacokinetic and hemostatic efficacies have usually been performed in the canine hemophilia models. Not only does this require substantially more purified protein, the sensitivity of the dogs to endotoxin requires that the protein be purified under conditions such that it remains endotoxin-free. Thus, cell cultures must be scaled up for purification in commercial-grade bioreactors either at great cost to the investigator or through research collaborations with interested parties within the biotechnology industry. Differences in research priorities and protection of technology interests may limit the evaluation of potential promising bioengineered variants.

Availability of small animal models

Access to small animal models for the hemophilias has greatly advanced the progress of preclinical development in this area. Mice are less expensive to maintain, have shorter gestational periods, and are easier to breed making their availability to investigators critical to performing preclinical evaluation of novel recombinant variants. FVIII exon 16 and exon 17 knockout (KO) mice have provided useful models for hemophilia A [69,70]. These targeted disruptions lie within regions that encode a portion of the A3 domain within the FVIII light chain. Thus, these FVIII KO mice may still synthesize the heavy chain of FVIII. This must be considered in the evaluation of immunogenicity and clearance of novel rFVIII within this model. Several investigators have generated hemophilia B murine models [71–73]. The mice in these models were created by the deletion of the promoter as well as a portion of the gene itself and produce no FIX antigen. However, most patients with hemophilia B have circulating defective FIX with detectable antigen, so these murine models do not fully mimic characteristics of the human disease. Recently, a mouse hemophilia B model was reported that expresses human FIX R336Q (a naturally occurring hemophilia B mutation) [74]. This model more accurately reflects the human hemophilia B condition and exhibits tolerance to human FIX making it an important model for immunogenicity and clearance studies of novel rFIX variants. A similar hemophilia A model that is tolerant to human FVIIII would also be useful for preclinical studies. Investigators have characterized useful hemostatic efficacy methods for both the hemophilia A and B murine models that aids in the evaluation of novel recombinant proteins. In addition, insights from the understanding of the murine immune system will advance the evaluation of the immunogenicity of novel rFVIII and rFIX variants.

Models for thrombogenicity testing

Recently, growing literature has established elevated FVIII levels as a significant risk factor for venous thromboembolic disease, yet the mechanism remains unclear [75–78]. The effect of varying FVIII levels on thrombin generation in an *in vitro*

coagulation system was investigated and demonstrated that the duration of the initiation phase of coagulation was reduced and the maximum thrombin generation increased only when FVIII levels were approaching 150% [79]. O'Donnell *et al.* [80] demonstrated that prothrombin fragments 1 + 2 and thrombin-antithrombin complexes were elevated in individuals with elevated FVIII levels, suggestive of increased basal thrombin generation. Chronic exogenous administration of FVIII may also contribute to thrombotic risk. Curiously, children with severe hemophilia A receiving FVIII via central venous catheter devices frequently develop deep venous thrombosis of the upper venous system [81].

There has been limited data investigating the thrombogenicity of elevated FVIII in animal models. In one study, rFVIII was administered intravenously to mice leading to FVIII plasma levels equivalent to 250% of normal human plasma [82]. A controlled mild injury was then inflicted on the carotid artery by irradiation with filtered green light in combination with intravenous injection rose bengal dye. Thrombus size at the injury site was significantly enhanced following FVIII administration compared with controls. In another recent report, FVIII KO mice were subjected to inferior vena cava (IVC) injury by short time compression and vein ligation [83]. None of the FVIII KO mice formed a thrombus in contrast to six of eight wild-type control mice. FVIII KO mice were then reconstituted with human FVIII by tail vein injection to > 1 U mL^{-1}, subjected to the same IVC injury and seven of nine mice tested formed thrombi similar to wild-type mice. In the presence of a specific human FVIII inhibitor preincubated with the infused human FVIII, only one of nine FVIII KO mice formed an IVC thrombus in response to IVC injury. These studies highlight the important influence FVIII levels can have on thrombosis. FVIII bioengineered for improved secretion efficiency, potency and stability raises concerns that these modifications may increase the thrombosis risk *in vivo*. Preclinical studies should include testing in animal models to analyze the thrombotic potential of these novel rFVIII variants. These studies would ideally evaluate thrombogenicity following transient exposure mimicking a bolus infusion as well as chronic/repeated exposure mimicking plasma correction as in gene therapy or routine prophylaxis. These *in vivo* thrombogenicity studies could be adapted to murine models of hemophilia B and hemophilia inhibitors to extend the preclinical studies of novel variants of rFIX and rVIIa as well.

New bioassays

As the functional properties of bioengineered recombinant clotting factors are altered, the current activity and antigen assays used for so long as surrogates for guiding therapy may no longer be useful. New bioassays will be necessary to evaluate these molecules both in preclinical studies but also to prepare for the transition to human clinical trials. Some existing alternative bioassays are being evaluated although their usefulness as surrogate markers for clinical efficacy has not yet been established.

Universal assays of coagulation Universal assays of the clotting system have been available for many years. Recently, in the search for assays to measure the effect of rVIIa and APCCs, there has been renewed interest in these universal assays. The rotational thromboelastogram analyses whole blood, measuring the effect of both the cellular and plasma components on clot formation [84]. This has demonstrated greater sensitivity than traditional clotting assays and allows the analysis of fibrin formation in real time. It measures the viscoelastic properties of blood induced to clot under low shear. Changes in clot elasticity are measured and reports various physicochemical properties of the clot. This type of analysis, if able to be standardized, could provide the type of surrogate assay for monitoring the effect of bioengineered forms of rVIIa. In addition this assay has enhanced sensitivity to very low levels of FVIII (at ranges 100–1000 times lower than can be measured by standard FVIII assays) [85]. This may be useful in defining residual FVIII or FIX activity for variants bioengineered for inactivation resistance or extended plasma half-life.

Thrombin generation assays The thrombin generation test was first introduced by Macfarlane and Biggs in 1953 to measure the thrombin-generating capacity of plasma [86]. In this method, defibrinated plasma samples were activated, then the thrombin formed was subsampled to a fibrinogen source to measure the amount of thrombin generated. Recently, Hemker and Beguin [87] have devised a modification of the thrombin generation test such that small peptides are used as the substrates for thrombin. Thrombin generation is initiated by a low concentration of TF added to a plasma sample and thrombin activity is measured continuously using a specific fluorescence peptide substrate that is cleaved by thrombin liberating a fluorophore. The rate of development of fluorescence intensity can be converted to a thrombin-equivalent concentration based on a reference curve. Many parameters can be measured including time to onset of thrombin generation, rate of thrombin burst generation, peak thrombin, time to peak thrombin, and the thrombin potential (area under the curve; a measure of the total thrombin formed after coagulation was triggered). This is also a test, which if it can be standardized, could become part of the clinical evaluation in hemophilia therapeutics. The thrombin potential may be particularly useful in more precisely defining the potency of bioengineered rFVIII with increased sensitivity to thrombin cleavage and inactivation resistance or similarly to monitor the effect of bioengineered rVIIa with increased potency. rFVIII variants with inactivation resistance may exhibit substantial thrombin potential even when plasma FVIII as measured by standard APTT or chromogenic assays has fallen to subtherapeutic levels.

Conclusion

The last 20 years have been remarkable for hemophilia care with advanced technologies to preserve the safety of clotting

factors from pathogens, the development of recombinant clotting factors and effective bypassing agents for patients with inhibitors, refinement of clinical assays of coagulation, a clinical cure through the implementation of prophylaxis from early childhood and the first steps toward a genetic cure via gene therapy strategies. Now, in the new millennium, we may see a partnering of these advancements for the next generation of patients. Novel bioengineered recombinant clotting factors may be utilized to liberate our patients from the adverse effects of their prophylactic management (the need for frequent venipuncture or central venous access devices, high costs), be monitored by new bioassays such as thrombin generation tests, or be partnered with gene therapy strategies to provide an ultimate cure. Indeed, a more immediate impact may be adapting these bioengineered recombinants for commercial production within mammalian cell lines or within transgenic livestock to produce low cost concentrates and increase their availability, particularly to the developing world. It is in these advances that the full promise of the recombinant era will be realized.

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