Hemophilia is caused by functional deficiency of a single coagulation protein. Absence of such proteins—factor VIII (FVIII) in hemophilia A and factor IX (FIX) in hemophilia B—may lead to spontaneous internal bleeding with joint damage, intracranial hemorrhage, and death. Since the 1840s, transfusion of whole blood has been used to treat hemophilia-associated bleeding. It wasn’t until 1911 that FVIII was detected in plasma and 1937 when its role was described in hemostasis and the coagulation cascade (Figure 1). These advances led to the development of plasma transfusions in the 1940s, plasma concentrates in the 1950s, cryoprecipitate in the early 1960s, and freeze-dried FVIII products for storage and home use in the late 1960s.

However, as use of blood products became more common, concerns began to arise about infectious contamination, highlighted by outbreaks of hepatitis C virus infection in the 1970s and human immunodeficiency virus (HIV) infection in the 1980s among hemophilia patients receiving pooled blood products. Concerns about infected blood products led to the development of recombinant FVIII (rFVIII; approved by the US Food and Drug Administration in 1992) and recombinant FIX (rFIX; approved in 1997). The use of these recombinant coagulation proteins has changed hemophilia care and increased the life-expectancy of patients with severe hemophilia from approximately 20 years in the 1970s to an essentially normal lifespan today. Long-term prophylactic factor replacement therapy also has reduced morbidity, decreasing the risk of joint damage and intracranial hemorrhage, and improved the quality of life for both children and adults with hemophilia. Recombinant factors are safe and provide independence from reliance on donor blood products.

However, use of recombinant factors is not without its challenges. These substances are expensive, costing more than $250,000 a year per adult in the United States. Because of their short half-life, they need to be administered every other day or several times a week, representing a significant time commitment and presenting problems with maintaining adherence. In addition, they require venous access, which often requires placement of a central venous access device (ie, port) and involves risks of sepsis and thrombosis. Finally, 25%–30% of hemophilia A and 3%–4% of hemophilia B patients develop alloantibodies to these recombinant factors, inhibiting their efficacy and resulting in renewed problems with bleeding. Whereas gene therapy is a potential solution, a cure is not yet available.

Thus, there is an urgent need for new, improved clotting factors to treat hemophilia patients and individuals with acquired bleeding problems due to administration of oral anticoagulants (eg, warfarin) who either have been given a supratherapeutic dose or have developed bleeding in conjunction with trauma and surgery. New oral anticoagulants, such as rivaroxaban and dabigatran, also present challenges to reversal and inhibition of bleeding events. Prothrombin complex concentrates (PCCs), fresh frozen plasma (FFP), anti-inhibitor coagulant complex

**Abstract**

The history of clotting factors is inextricably tied to that of hemophilia. The development of recombinant factor VIII (rFVIII) and factor IX (rFIX) in the 1990s has resulted in hemophilia patients having a virtually normal lifespan and significantly fewer complications, such as joint and intracranial bleeding, following prophylactic infusions. However, these treatments are limited by the short-half lives of rFVIII and rFIX, meaning that patients must endure three- or four-times-weekly infusions. This report summarizes some of the exciting advances in the management of hemophilia that have been reported in recent years. They include the addition of polyethylene glycol (PEGylation) or fusion with another protein, such as immunoglobulin G or albumin, to decrease the frequency of infusions, provide prolonged protection from bleeding, limit the need for central venous access devices, and encourage patients to transition to a prophylaxis regimen. In addition, advances have been made in improving the safety and efficacy of long-lasting rFVIII proteins for hemophilia A and the development of alternative agents to treat hemophilia and anticoagulant-related bleeding (eg, warfarin reversal) and new oral anticoagulants (eg, direct thrombin or factor Xa inhibitors).
MOLECULAR APPROACHES FOR FVIIa

T H E 	 H E M O P H I L I A 	 R E P O R T

Anthony Sung, MD
Advances in Clotting Factors: From Bench to Bedside

Although the development of recombinant factors in the 1990s represented a tremendous advance in the treatment of hemophilia, efforts have been underway to circumvent problems associated with their short half-life and frequent dosing. Research teams have reported on the structure of FVIII and FIX and their mechanisms of action, allowing engineering of variants with improved half-lives. These methods include conjugation with polyethylene glycol (PEG) in a process known as PEGylation, use of PEGylated liposomes as a mechanism for sustained release, fusion with the crystallizable fragment (Fc) of the constant region of immunoglobulin (Ig) or albumin, and novel modifications to clotting factors. Although PEGylated liposomes have failed to demonstrate in vivo efficacy,\textsuperscript{10-12} other methods have shown considerable promise.

**PEGylation**

PEGylation is achieved by covalently attaching PEG to residues on target proteins such as lysine or N-terminal amines. However, random PEGylation may reduce the activity of the protein, and product heterogeneity may result in inconsistent effectiveness.

More recently, site-directed PEGylation via attachment of PEG-maleimide to cysteine residues has improved results with many proteins, including tumor necrosis factor-alpha, monoclonal antibody Fab fragment, vascular endothelial growth factor-aptamer, epoetin beta, and interferon alfa.\textsuperscript{13,14} In this approach, missense mutations are introduced at surface residues of FVIII to incorporate cysteine residues for conjugation with PEG-maleimide. This allows selective modification at the desired location, so PEGylation does not interfere with protein function and ensures product homogeneity. To date, no long-term safety concerns have arisen with this method, including with PEGylated FVIII products developed by Bayer Healthcare (BAY 94-9027), Baxter (BAX 855), and Novo Nordisk (N8-GP).

Of note, PEGylated products may vary by site of PEGylation and type of PEG used. PEG molecules also may differ in size, with smaller molecules being more rapidly cleared than larger ones. In addition, tissue penetration may vary, with smaller molecules having greater permeability. With PEG molecules > 10 kDa, pinocytotic uptake into macrophages and Kupffer cells is increased; with PEG molecules > 30 kDa, kidney clearance is decreased; and with PEG molecules > 50 kDa, liver clearance is increased.\textsuperscript{15,16} Therefore, the pharmacokinetics and pharmacodynamics—and safety and efficacy—of different PEG solutions vary.

In addition to improving half-life, PEGylation may reduce immunogenicity. PEGylation of L491C in the A2-domain of FVIII resulted in reduced inhibitory activity.\textsuperscript{17} This finding has been supported by other studies with different PEGylated proteins.\textsuperscript{15,18} PEGylation also may limit the inhibitory effect of alloantibodies.

**Fusion Proteins**

By covalently fusing coagulation factors with proteins having a much longer...
long-lasting recombinant factor VIII proteins for hemophilia A

Advances in clotting factors: From bench to bedside

Anthony Sung, MD

Based on a presentation by Amy D. Shapiro, MD, Chief Executive Officer and Co-Medical Director, Indiana Hemophilia Treatment Center, Indianapolis, Indiana.

PEGylation and fusion of Ig or albumin to FVIII and FIX are exciting strategies for increasing the half-life of these coagulation factors that are now being tested in phase 1–3 clinical trials. This section will review these developments.

Clinical Studies of PEG-FVIII Conjugates

Three PEG-FVIII conjugates currently are being tested in clinical trials: B-domain deleted recombinant FVIII (PEG-BDD-rFVIII; BAY 94-9027; www.clinicaltrials.gov ID No. NCT01184820), PEGylated full-length rFVIII (BAX 855; www.clinicaltrials.gov ID No. NCT01736475), and glycol-PEGylated rFVIII (N8-GP; www.clinicaltrials.gov ID No. NCT01480180).

PEGylation can impair a protein’s activity if bound to the wrong site, so it is important that this link is produced in such a way that it maintains the protein’s original function. One method of ensuring site-specific PEGylation is site-specific mutagenesis, which introduces cysteine mutations on the surface of B-domain deleted FVIII. Bayer HealthCare used this method in designing BAY 94-9027: PEG was conjugated to surface-exposed cysteines of rFVIII to retain full in vitro activity and vWF binding.

BAY 94-9027 was evaluated in a phase 1 study of 14 patients with severe hemophilia A. Seven patients were given 25 IU/kg twice weekly, and the other seven received 60 IU/kg once weekly. BAY 94-9027 was well tolerated and effective without causing serious adverse events or immunogenicity. The half-life was 19 hours, representing a 1.6-fold increase over the half-life of standard rFVIII (approximately 12 hours). Phase 2/3 studies are underway.

An alternative strategy was employed by Baxter scientists in the design of BAX...
This conjugation process resulted from the combination of an activated PEG reagent with accessible amino groups on FVIII; it was optimized to target and modify mainly the e-amino groups of lysine residues. BAX 855 reportedly retains all physiologic properties of FVIII except binding to the low-density lipoprotein receptor-related protein clearance receptor; preclinical testing revealed normal activity and a prolonged half-life when compared with unmodified rFVIII. N8-GP is a rFVIII with site-directed glycoPEGylation being developed by Novo Nordisk. It is synthesized in a Chinese hamster ovary cell line with a truncated B domain of 21 amino acids; the terminal sialic acid on an O-glycan structure in the truncated B-domain is replaced by a conjugated sialic acid containing a branched 40-kDa PEG, resulting in a protein with a single medium-weight PEG attached to the B-domain. When the coagulation system is activated, thrombin cleaves the B-domain with the attached PEG, resulting in activated FVIII.

N8-GP was evaluated in a dose-escalation study (25, 50, or 75 IU/kg/dose) in 26 previously treated patients with severe hemophilia A. It was well tolerated at all dose levels, and no patient developed an inhibitor or binding antibodies to FVIII or N8-GP. N8-GP exhibited a dose-linear pharmacokinetic profile with a mean half-life of 19 hours (range, 11.6–27.3 hours), representing a 1.6-fold increase over that of standard rFVIII. Clearance was reduced by 30%, and the volumes of distributions were similar to those of standard products. N8-GP currently is being tested in phase 3 clinical trials. A similar product for hemophilia B, N9-GP (glycoPEGylation of rFIX), also has shown promise in clinical trials.

**Fc Fusion Proteins**

rFVIIIFc was developed by Biogen Idec by fusing a single B-domain deleted rFVIII that is produced from human embryonic kidney cells to the dimeric Fc receptor (FcRn) knockout mice, supporting the role of the Fc fragment and interaction with FcRn in protecting the fusion protein from degradation.

A phase 1/2 study of rFVIIIFc in 16 patients with severe hemophilia who were given either 25 or 65 IU/kg rFVIII followed by an equal dose of rFVIIIFc showed a 1.5- to 1.7-fold increase in mean half-life (18.8 hours for both doses) for rFVIIIFc over that of rFVIII (12.2 hours for the lower dose and 11.0 hours for the higher dose). Both products had similar dose-dependent peak plasma concentrations. No drug-related adverse events, inhibitors, or severe bleeding was observed.

A phase 3, multicenter study of rFVIIIFc (A-LONG, www.ClinicalTrials.gov identifier NCT01458106) was completed recently. Treatment arms included individualized prophylaxis at 3- to 5-day intervals, weekly prophylaxis, and episodic (on-demand) treatment. Preliminary results from this study were presented by Mahlangu and coworkers at the 65th Annual Meeting of the National Hemophilia Foundation in October 2013 and are summarized by Dr. Holleh D. Husseinzadeh elsewhere in this edition of The Hemophilia Report. Once the data from this trial are fully compiled and analyzed, the study should provide valuable information on the safety and effectiveness of different strategies for prophylaxis of hemophilia A and on-demand treatment of bleeding episodes with rFVIIIFc. A similar study is ongoing in children (Kids A-LONG, www.ClinicalTrials.gov identifier NCT01458106).**

**Summary**

PEGylation and Fc fusion are exciting strategies that may prolong the half-life of rFVIII; extend dosing intervals; and potentially improve compliance, access, and safety. Both PEGylated and Fc fusion products have a half-life of 18–19 hours, whereas the half-life of vWF also is 18 hours. Given that vWF is needed to stabilize and protect FVIII, it is possible that the half-life of vWF may represent a new limit to how far the half-life of VIII products may be extended. This remains a significant improvement over conventional products, and the final results of phase 3 studies such as A-LONG are eagerly anticipated.

**THE OLD AND THE NEW: PCCs, rFVIIa, AND LONG-LASTING COAGULATION PROTEINS**

Based on a presentation by Margaret V. Ragni, MD, MPH, Professor of Medicine, Division of Hematology/Oncology, University of Pittsburgh, and Director, Hemophilia Center of Western Pennsylvania, Pittsburgh, Pennsylvania.

PCCs contain combinations of clotting factors and proteins C and S. Four-factor PCCs (eg, Beriplex, Octaplex, Kcentra) contain factors II, VII, IX, and X, whereas three-factor PCCs (eg, Behbunin, Profilnine) contain factors II, IX, and X but little VII. These products initially were developed as bypassing agents to treat hemophilia patients with inhibitors to FVIII or FIX. These factors act downstream of FVIII and FIX (Figure 1), bypassing their activity. FEIBA is a formulation of PCCs that has activated clotting factors to enhance hemostasis. PCCs and FEIBA start working within minutes and carry a low risk of infectious transmission due to viral inactivation by filtration, nanofiltration, pasteurization, or solvent detergent treatment.

The substance known as rFVIIa also

855, a 20-kDa PEGylated full-length rFVIII. This conjugation process resulted from the combination of an activated PEG reagent with accessible amino groups on FVIII; it was optimized to target and modify mainly the e-amino groups of lysine residues. BAX 855 reportedly retains all physiologic properties of FVIII except binding to the low-density lipoprotein receptor-related protein clearance receptor; preclinical testing revealed normal activity and a prolonged half-life when compared with unmodified rFVIII. This study should provide valuable information on the safety and effectiveness of different strategies for prophylaxis of hemophilia A and on-demand treatment of bleeding episodes with rFVIIIFc. A similar study is ongoing in children (Kids A-LONG, www.ClinicalTrials.gov identifier NCT01458106).
was developed as a bypassing agent to treat hemophilia-associated bleeding. It may produce a “thrombin burst” via activation of FIX, FX, and FII on the surface of activated platelets. Like PCCs and FEIBA, rFVIIa is expensive, and its use carries a significant risk of thrombosis.

To assess the efficacy of these hemostatic strategies, quantitative and qualitative laboratory measures of clot formation are needed. These analytic tools include the thrombin generation assay (TGA), thromboelastography (TEG), and rotational thromboelastometry (ROTEM), which provide a more comprehensive assessment of clot formation than do such standard assays as prothrombin time (PT) and activated partial thromboplastin time (aPTT).41 Important parameters of TGA include the lag time (the time to initiation of thrombin generation) and endogenous thrombin potential (area under the curve). Those of TEG include the rate of clot formation and strength and stability of the clot. For ROTEM, important parameters include the time to clot formation (clotting time), maximum clot firmness, and time to clot lysis.

**PCCs and rFVIIa in Surgery and Trauma (With or Without Warfarin)**

Preclinical studies in porcine liver laceration and spleen injury models provide in vitro and in vivo evidence that PCCs are more effective than rFVIIa in restoring thrombin generation and reducing blood loss.42,43 These results are supported by clinical studies of coagulopathic patients undergoing surgery with excessive bleeding requiring PCCs or rFVIIa.

In a study of patients undergoing cardiopulmonary bypass, three-factor PCCs (18.9–30.9 U/kg) reduced transfusion requirements to a greater degree than did rFVIIa (90–120 µg/kg).44 Another comparison study of three-factor PCCs (25 U/kg) with rFVIIa (90 µg/kg) in 85 traumatic brain injury patients revealed significantly greater reductions in red blood cell (RBC) and FFP requirements and a lower mortality among the PCC group.45 In a randomized trial of patients with acute major surgical hemorrhage who also received vitamin K, four-factor PCCs were superior to FFP; whereas other studies showed that the combination of FFP and PCCs may yield even better results.46,47

At the same time, in coagulopathic trauma patients (half of whom were receiving warfarin), administration of three-factor PCCs (25 U/kg) rapidly corrected the international normalized ratio (INR) and reduced the RBC requirement; however, there was no survival benefit, as with most studies of rFVIIa in trauma.48 Controversy remains regarding rFVIIa use, given its high cost, lack of dosing guidelines, and thrombosis risk.

Another challenge of using PCCs and rFVIIa is determining the proper dose. Some studies have used algorithms that adjusted the dose based on INR (eg, three-factor PCC dosed at 25 U/kg for an INR of 2.0–3.9, 35 U/kg for an INR of 4.0–6.0, and 50 U/kg for an INR > 6.0), whereas others have used ROTEM testing to guide PCC therapy, and still others have used fixed doses.49 The optimal timing and frequency of administration also are unclear.

Of note, four-factor PCCs appear to be more effective than are three-factor PCCs.47 This may be due to consumption of FVII in cases of extensive surgery or trauma, bleeding, or warfarin use and the fact that three-factor PCCs are poor in FVII. When the INR > 6.0, three-factor PCCs may have little efficacy.50,51 Interestingly, in cases of warfarin reversal for acute bleeding, administration of 10–90 µg/kg of rFVIIa rapidly corrected the INR; unlike with four-factor PCCs, however, it seemed to do little to reduce bleeding.52 In parallel with these findings, ROTEM clot stability and clot lysis time in warfarin-treated patients appeared to improve more after treatment with PCCs than after use of rFVIIa.53

**PCCs, rFVIIa, and the New Oral Anticoagulants**

New oral anticoagulants (thrombin and factor Xa inhibitors) have many advantages over warfarin, including no requirement for monitoring, few drug–drug interactions, and lower bleeding rates. However, reversal of these agents for life-threatening bleeding is complicated by the absence of an effective antidote. The half-life and duration of action of new oral anticoagulants are short. However, acute bleeding often occurs, and waiting for the drug to wear off is unacceptable.

Unfortunately, there is little evidence of the best approach to stop bleeding in patients on new oral anticoagulants. In preclinical studies, FEIBA, PCCs, and rFVIIa improved parameters such as bleeding time, PT, and aPTT, but there was poor correlation with the amount of blood loss.54,55 The best results appeared to follow the use of high-dose four-factor PCCs (eg, 50 U/kg) and FEIBA, with rFVIIa and FFP having little effect.56,57 Studies in healthy human volunteers appeared to support these findings, with reversal of abnormal TEG and ROTEM results seen with FEIBA (20–120 U/kg) and four-factor PCCs (50 U/kg); use of rFVIIa (20–120 µg/kg) was less effective.58 However, few data for these products exist in bleeding patients.

Novel antidotes to reverse new oral anticoagulants are being developed. They include an Xa congener that neutralizes Xa coagulation inhibitor function59 and a dabigatran-specific antidote (aDabi-Fab) that mimics thrombin structure (but not function) and binds to dabigatran with 350-fold greater avidity.60 These agents are still in the exploratory stage.

**Treatment Considerations**

To reverse warfarin-related bleeding or surgical and trauma-related bleeding, PCCs appear to be superior to rFVIIa and warfarin.61 The combination of PCC and FFP or PCC and rFVIIa may correct laboratory abnormalities such as INR even more rapidly,62 yet extreme caution must be exercised, given the significant thrombotic risk of each agent, and recommendations regarding combination therapy must await further studies. Furthermore, use of these agents should be avoided in patients with recent (< 3 months) thromboembolism.63 Even in the absence of thromboembolism, dosing should be judicious (eg, 25 U/kg of four-factor PCC for an INR of 2.0–3.9, 35 U/kg for an INR of 4.0–6.0, and 50 U/kg for an INR > 6.0).50,62 The lowest effective...
dose of rFVIIa has not been established. Regarding new oral anticoagulants, it is unclear whether PCCs or rFVIIa can reverse their effects. Caution should be exercised, since there apparently is little correlation between correction of laboratory abnormalities and reversal of bleeding, and dosing and monitoring are not established.\textsuperscript{8,63–65} New antides are under development but are experimental at this time. Transfusion support and surgical hemostasis should be provided, if indicated. Of note, dialysis with activated charcoal may be effective for dabigatran if initiated within 2–4 hours of ingestion; however, this may not be effective to reverse the effects of rivaroxaban, which is highly protein bound.\textsuperscript{65,66}

\section*{CONCLUSION}

Tremendous advances have been made in hemostasis over the past several years. Advances in clotting factors (eg, PEGylation, Ig or albumin fusion with FVIII or FIX) promise to improve hemophilia treatment by increasing factor half-life, decreasing the frequency of infusions, and potentially improving compliance and access while decreasing the risk of bleeding complications. Other novel agents (eg, TFPI, AT3 inhibitors) are also very exciting. Meanwhile, FEIBA, PCCs, and rFVIIa may help decrease life-threatening bleeding for hemophilia patients and those on warfarin or new oral anticoagulants who experience surgical or traumatic bleeding. As with all these agents, caution must be exercised, given the risk of upsetting the balance between hemostasis and thrombosis. Nonetheless, the coming years promise major advances in clotting factor treatments.

\section*{REFERENCES}


52. Rosovsky RP, Crowther MA. What is the evidence for the off-label use of recombinant factor VIIa (rFVIIa) in the acute reversal of warfarin? Hematology Am Soc Hematol Educ Program. 2008:36–38.


