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Review Article

Recombinant factor VIII: past, present and future of treatment of hemophilia A

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Summary

The development of recombinant factor VIII (rFVIII) was initially driven by the necessity to treat hemophilia A (HA) patients with FVIII concentrates without the risk of transmitting infectious agents. Over the last three decades the safety of rFVIII has been further improved by completely removing animal or human proteins from the manufacturing process, so that patients would not be exposed to known or emerging pathogens. Recent efforts have concentrated on improving the expression of rFVIII, reducing its immunogenicity and enhancing its pharmacokinetic (PK) behavior. These new goals have been possible thanks to the development of biotechnology and a better knowledge of the function and structure of FVIII. Several approaches such as deletion of the B-domain, expression of FVIII by human cell lines, sequence modification, structural modification, coexpression with other proteins, fusion with the Fc fragment of immunoglobulins

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and PEGylation have been utilized. As a result of these efforts, different rFVIII products have been validated in terms of efficacy, immunogenicity and PK profile. Other technologies are currently being explored to improve the PK of FVIII and allow its subcutaneous administration. Although nonreplacement therapies and HA gene therapy appear to be promising alternatives for HA, rFVIII will very likely remain as a critical component for the treatment of HA because of its physiological activity and mode of action, as well as its unique ability to induce or restore tolerance to exogenous FVIII. This review summarizes the principal features of past, current and emerging rFVIII products for HA.

Key words: Hemophilia A – Coagulation factor disorders – Recombinant FVIII – Standard half-life FVIII – Extended half-life FVIII

Introduction

Hemophilia A (HA) and B are congenital X-linked bleeding disorders resulting from a deficiency of clotting factor VIII (FVIII) and IX (FIX), respectively (1). HA affects 1 in 5,000 male births and it is characterized, according to the basal FVIII level, by spontaneous or traumatic bleeding episodes, which can progress to debilitating arthropathy and significantly impact patients' quality of life (1).

The primary aim of care is to prevent and treat hemorrhagic episodes using replacement therapy with FVIII concentrates, given on demand or prophylactically (2). Recombinant FVIII (rFVIII) products represent a major advance over the past century for the treatment of HA in terms of safety, hemostatic efficacy and manufacturing. The development of neutralizing antibodies, or inhibitors, against exogenous FVIII and the limited half-life (~12 hours) of many available products remain major challenges (3, 4).

History of rFVIII Products

FVIII replacement therapy has been the cornerstone in the treatment of hemophilia and has made major advances over the past century from the administration of blood transfusions to the use of recombinant products in 1992 (5). Prior to the 1950s, whole blood transfusion was the only possible therapy. Subsequently, in the 1960s it was discovered that a fraction cryoprecipitated from plasma contained large amounts of FVIII (6). Purified plasma-derived (pd)FVIII concentrates have been available since the 1970s, and in the following 10 years fractionation and purification processes evolved making plasma-derived concentrates available and allowing home replacement therapy (5).

During the first part of the 1980s, the HIV epidemic devastated the hemophilia community, and in the late 1990s over 95% of severe HA patients had been infected by hepatitis C virus through pdFVIII (5). As a consequence, many measures including enhanced donor screening tests and the introduction of viral inactivation have been implemented to improve the infectious safety of plasma-derived concentrates. Nevertheless, the fear of transmitting other unknown, not yet identified and emerging infectious diseases and the desire for safer and more convenient therapies have led to an increasing interest in recombinant DNA technology (7, 8).

In 1984 the FVIII gene was cloned and led to the licensure of rFVIII in 1992 (5). rFVIII products, which are derived through heterologous transfection of rFVIII DNA plasmids into a nonhuman mammalian cell line, virtually eliminated blood-borne pathogen transmission (9). The introduction of rFVIII concentrates nearly 20 years ago represented a significant advance in the treatment of HA. The most challenging aspects of HA management today are the occurrence of FVIII inhibitors and the burden of frequent intravenous infusions required for prophylactic replacement therapy (5).

Main Features of rFVIII

The human FVIII (hFVIII) gene is localized on the long arm of the X chromosome and its expression is predominantly in sinusoidal and vascular endothelial cells in the liver and the lungs (10, 11). After translation, the protein undergoes further processing of N-linked glycans, sulfation of specific tyrosine residues, copper binding, O-linked glycosylation and intracellular proteolysis within the Golgi apparatus and endoplasmic reticulum prior to secretion into plasma (12, 13). The mature FVIII is a complex 300-kD heterodimeric glycoprotein consisting of six structural domains and three acidic subdomains,

organized in a heavy chain [A1(a1)A2(a2)B] and a light chain [(a3)A3C1C2] (12).

rFVIII is one of the largest and most complex proteins manufactured commercially to date (14). Because of its structure and post-translational modification requirements, production of rFVIII depends on the use of mammalian cell lines, and either Chinese hamster ovary (CHO) or baby hamster kidney (BHK) cells were initially used. In fact, both the CHO and BHK cell lines have demonstrated their capacity to synthesize rFVIII that includes all of the post-translational modifications necessary for its hemostatic activity (9, 14).

The expressed proteins are secreted into a culture medium, resulting in a large-scale production of rFVIII products, which in some cases contain human and animal proteins for stabilization (15, 16). The purification process of rFVIII involves many steps, such as multiple chromatography columns, including immunoaffinity and cation exchange (17), followed by a solvent/detergent step, and either ultra- or nanofiltration to reduce transmission of enveloped (i.e., HIV, hepatitis B and hepatitis C virus) and nonenveloped viruses (i.e., hepatitis A virus and parvovirus B19) (9).

Conventional rFVIII concentrates behave similarly to pdFVIII with regard to biochemical and hemostatic properties. In fact, both proteins have similar pharmacokinetic (PK) profiles and cleavage patterns in vitro (18). However, minor differences in the post-translational modifications are observed in FVIII molecules derived from different species and cells lines (19). For example, carbohydrate structural differences specific to the expression cell lines are present; for instance CHO cell lines can synthesize the glycan epitope *N*-glycolyl neuraminic acid (Neu5Gc) moiety, which is not present in the endogenous FVIIIs (13). Similarly, BHK cells synthesize Galα1 →3Gal groups onto some of their polyantennary sugar chains, which are not normally present in human or primate glycoproteins (19). The expression of Galα1 →3Gal groups has been suggested to be a potential source of immunogenicity. These cell lines have been the only source of rFVIII products with good clotting efficacy and excellent viral safety for more than 30 years (13, 15). However, newer products have been recently made using human embryonic kidney (HEK) cells (13). This HEK-derived rFVIII product (human-cl rhFVIII) does not contain Galα1 →3Gal and Neu5Gc epitope (19).

Sulfation is another post-translational modification important for the function of FVIII (20). Six active sulfation sites of tyrosine residues have been identified in hFVIII and all six sulfation sites are necessary for the full procoagulant activity of FVIII. Sulfation of the residue Y1680 (Tyr 1680) is considered crucial for the stability of FVIII and its binding to von Willebrand factor (VWF) (13). The proportion of Tyr 1680 is high in BHK-derived rFVIII products as well as in other rFVIII products made from different cell lines, ranging from 1% to 6.5% for second-generation rFVIII to up to 15% for third-generation rFVIII (21).

Generations of rFVIII

Several preparations of rFVIII are currently available for patients with HA (5). Manufacturing of rFVIII evolved over decades and provided products that were classified depending on whether animal-derived or human-derived proteins were used during production and were present in the final formulation (22).

rFVIII products can be divided in the following three different generations: i) first-generation products using animal-derived proteins in the cell culture medium and human serum albumin in the final formulation to stabilize FVIII; ii) second-generation products using human-derived proteins in the culture medium but with no albumin added in the final formulation; and iii) third-generation products manufactured without addition of animal or human proteins during processing or in the final formulation (21) (Table I).

First-generation rFVIII products (Kogenate; Recombinate) contain both animal- and human-derived proteins in the cell culture as well as human albumin added in the final formulation as stabilizers (21). To improve safety, second-generation rFVIII concentrates (Kogenate FS; Helixate FS) have removed human albumin and have used sucrose as a stabilizing agent since 2000. Moreover, the human serum albumin used in the cell culture process has been removed prior to the final formulation. Third-generation products (Advate; ReFacto), which have been licensed

Table I. *Characteristics of first developed recombinant FVIII products.*

Generation	Product (manufacturer)	FVIII	Cell line	Culture medium	Stabilizer	Purification/viral inactivation
First	Recombinate (Shire)	Full-length	CHO	Bovine serum albumin	Human albumin	IAC/IEC
Second	Kogenate FS (Bayer)	Full-length	BHK	Human plasma protein solution	Sucrose	IAC/IEC/SD/UF
Second	Helixate FS (CSL Behring)	Full-length	BHK	Human plasma protein solution	Sucrose	IAC/IEC/SD/UF
Third	Advate (Shire)	Full-length	CHO	None	Trehalose	IAC/IEC/SD
Third	ReFacto (Pfizer)	B-domain deleted	CHO	None	Sucrose	IAC/IEC/SD/NF

BHK, baby hamster kidney; CHO, Chinese hamster ovary; FVIII, factor VIII; IAC, immunoaffinity chromatography; IEC, ion exchange chromatography; NF, nanofiltration; SD, solvent/detergent treatment; UF, ultrafiltration.

since 2003, do not use any human or animal proteins in the cell culture or the production of FVIII products (21, 22).

rFVIII concentrates can also be divided into full-length (FL) or B-domain-deleted (BDD) concentrates, in which the B-domain is removed from the FVIII molecule (21). Early on in the study of rFVIII expression, it was demonstrated that the B-domain of FVIII could be removed without loss of FVIII final clotting efficacy (18). This modification significantly improved secretion from the cell in the recombinant process, and thus BDD-FVIII products such as ReFacto were developed (23). In order to show the efficacy of BDD-FVIII, direct comparison studies were performed using BBD-FVIII (ReFacto) and FL-rFVIII (Advate). The direct PK comparisons showed that the FVIII products were bioequivalent to each other (24).

Conventional rFVIII concentrates differ in protein sequence or manufacturing process but offer patients similar PK and security profiles (17). Clinical studies have shown that the recombinant coagulation factors provide safe, well-tolerated and effective treatment of HA whether used in on-demand therapy, prophylactic regimens or surgery (15). To date, there is no consensus regarding whether any one of the currently available rFVIII products has significant clinical advantage over the others. Each product has a strong safety record and has been available for a number of years in many countries. Moreover, there are many studies in patients with HA but randomized clinical trials comparing one generation with another product or generation have not been conducted and are unlikely to take place in the future (25).

Standard Half-life rFVIII Products

Since 2013, the U.S. Food and Drug Administration (FDA) has licensed four additional third-generation rFVIII concentrates with standard half-life (17). Each of these products has been distinguished from other available factor concentrates by changes in cell line, protein structure or manufacturing techniques (Table II). The first of these new rFVIII products to be approved by the U.S. FDA is turoctocog alfa (NovoEight), a B-domain truncated rFVIII produced in CHO cell lines without addition of any human- or animal-derived materials. Its primary structure (called N8 protein) contains a B-domain that is truncated to 10 amino acids from the N-terminus and 11 amino acids from the C-terminus, and shows sulfation and glycosylation patterns very similar to those of pdFVIII (26). Glycosylation of N8, as for the pdFVIII, is mainly characterized by the presence of four N-linked glycosylations and one O-linked glycosylation (Ser750) in the B-domain (26, 27). Moreover, nonhuman glycan epitopes, critically expressed by different culture cells, are absent (16). As mentioned above, it has been hypothesized that these nonhuman epitopes carry potential implications for immunogenicity (13).

Two phase III trials showed the safety and efficacy of turoctocog alfa in previously treated patients (PTPs)

Table II. *Characteristics of new standard half-life recombinant FVIII products.*

Brand name (manufacturer)	Generic name	Frequency of i.v. administration	Mean ABR (95% CI or ± SD)	Median ABR (IQR or range)	References
NovoEight (Novo Nordisk)	Turoctocog alfa	3 × weekly or q2d	6.5 (5.3-8.0)	3.7 (8.7)	(28-31)
Nuwiq (Octapharma)	Simoctocog alfa	3 × weekly or q2d	2.3 (± 3.7)[a] 4.1 (± 5.2)[b]	0.9 (0-14.7)[a] 1.9 (0-20.7)[b]	(42, 43)
Kovaltry (Bayer)	Octocog alfa	2 × weekly 3 × weekly 2 × weekly 3 × weekly	3.8 (± 5.2) 4.9 (± 6.8)	1 (0.0-8) 2 (0.5-5) 4 (0.0-8) 2 (0.0-4.9)	(32-34)
Afstyla (CSL Behring)	Lonoctocog alfa	2-3 × weekly	3.1 (± 5.1)	1.14 (0.0-4.2)	(38-40)

[a]Adult patients.
[b]Pediatric patients.
IQR, difference between 75th percentile (3rd quartile) and 25th percentile (1st quartile); ABR, annual bleeding rate; CI, confidence interval; SD, standard deviation; q2d, every 2 days.

in the prevention and treatment of hemorrhagic episodes: Guardian 1 in adult/adolescent patients and Guardian™3 in pediatric patients (28, 29). Interim results from Guardian 2 showed a favorable long-term effect of turoctocog alfa on annualized bleeding ratio (ABR) (30, 31). No FVIII inhibitors were detected. The Guardian clinical trial program is still ongoing with Guardian 4 (ClinicalTrials.gov Identifier NCT01493778), a phase III trial in pediatric previously untreated patients (PUPs) with HA (30).

In 2016, octocog alfa (BAY-81-8973, Kovaltry) was FDA-approved on the basis of the "Long-Term Efficacy Open-Label Program in Severe Hemophilia A Disease" (LEOPOLD) clinical trial program LEOPOLD I (32), LEOPOLD II (33) and Kids trials (34). It is an unmodified FL-rFVIII produced in BHK cell lines with the same amino acid sequence as its predecessor (Kogenate FS), but with advanced manufacturing technologies. The changes in its production consist in coexpression of heat shock protein 70 (HSP70) in the new cell bank and production without addition of human- or animal-derived materials to the cell culture, purification or formulation processes (35). These changes led to differences in sialylation of the rFVIII product that is characterized by a high degree of sialic acid capping of N-terminal glycans on the molecular surface. This new sialylation profile is believed to be responsible for the 10% longer half-life of Kovaltry when compared with Kogenate FS in crossover PK studies (35). Kovaltry showed a low ABR when used as prophylaxis in PTPs (adult and pediatric) with severe HA (33, 34). Infusion of the product was generally well tolerated, with no inhibitor development reported in PTPs.

Lonoctocog alfa (rVIII-SingleChain, CSL-627, Afstyla) is the only rFVIII with a specific single-chain design which increases its stability during manufacturing and displays a stronger affinity for VWF (36). It is produced in CHO cells without the use of human- or animal-derived proteins, and purified using chromatographic techniques (37). The characteristic design of this new molecule consists of a single chain in which the heavy and light chains are covalently liked by a truncated B-domain. This structure differs from other rFVIII preparations, as these have two-chain molecules that are linked via a labile metal–ion bridge (36). Even if VWF exerts a protective effect on FVIII from degradation and clearance, the faster and tighter binding of lonoctocog alfa to VWF determines only a slight prolongation of its half-life when compared to octocog alfa (14.5 vs. 13.3 hours) (38, 39). It is to be noted that one-stage assays (OSAs) underestimate FVIII procoagulant activity (FVIII:C) levels in patients receiving lonoctocog alfa by approximately 45-50%, whereas chromogenic substrate assays (CSAs) are closer to target. The underestimation affecting the calculation of FVIII activity with lonoctocog alfa in OSAs could be predictable and corrected by multiplying the results by a conversion factor of 2 (36). Safety and clinical efficacy of the molecule were demonstrated in the AFFINITY program, which consists of

two pivotal studies (one with an adult/adolescent population and the other with a pediatric population, both completed), as well as an ongoing extension study (subjects 65 years or younger) to assess long-term safety data (40).

Finally, simoctocog alfa (human-cl rhFVIII, Nuwiq) is the first BDD-rFVIII produced in HEK cell lines. It was approved by the FDA in 2015. Manufacturing of HEK-derived products like simoctocog alfa, as mentioned above, ensured a human pattern of post-translational modifications (41). Its post-translational modifications (full sulfation of Tyr 1680) increased the VWF-binding affinity (16). The impact of post-translational modifications on immunogenicity is being investigated in the NuProtect study (GENA-05, ClinicalTrials.gov Identifier NCT01992549), which is still ongoing. Preliminary data results from an interim analysis demonstrated low inhibitor rates, with the cumulative incidence being 20.8% (95% confidence interval [CI], 10.68-30.95) for all inhibitors and 12.8% (95% CI, 4.49-21.25) for high-titer inhibitors (42). Several phase I/II and III trials have demonstrated the safety and efficacy of simoctocog alfa in the prevention and treatment of bleeds in PTPs with severe HA (43).

Extended Half-life rFVIII Products

Over the last 30 years the main goal in developing new treatments for hemophilia has been to increase the safety of concentrates with respect to viral and other infectious agent transmissions. Recently, due to the improvements in safety obtained by recombinant manufacturing of FVIII, the attention has shifted to modifying the FVIII molecule to improve replacement therapy. In particular, attempts were made to obtain FVIII products with extended half-life (EHL-FVIII) and enhanced functional activity, and to decrease immunogenicity (44). Researchers have assessed several protein modification techniques to obtain FVIII products with a prolonged half-life with the purpose of supporting an extension of the treatment intervals required for effective prophylaxis or higher trough levels with a standard number of infusions (17).

Among these newer techniques, to date only two approaches have led to regulatory approval: i) covalent attachment of FVIII to polyethylene glycol (PEG; PEGylation) and ii) fusion to the Fc portion of the IgG1 molecule (45). An alternative approach is modification with polysialic acid technology (17), which leads to interference of the receptor-mediated clearance (46). BAX-826 (Shire) is a polysialylated FL-rFVIII that was in phase I clinical trials for the treatment of HA (17), but its development was discontinued by Shire as the principal objective in the clinical trials was not achieved. So far, fusion of FVIII to albumin has been unsuccessful in preserving effective coagulation activity.

The findings obtained with the current EHL-FVIII products have been marked by a limited expansion of their half-life that is only 1.5- to 1.7-fold longer than that of wild-type factor (44) (Table III). On the other hand, the current status of FIX long-acting products is at the moment more attractive since the prolongation of their half-life is as great as 4- to 5-fold (44).

PEGylation

PEGylation consists of a covalent link between PEG (a hydrophilic inert substance) and a protein. The molecular weight and the size of the PEGylated protein are increased leading to reduced renal elimination, providing decreasing receptor-mediated clearance and steric shield against immunological recognition (46). While questions have been raised about retention of PEG in the reticuloendothelial system, the safety of PEG and licensed therapeutics using PEG technology has been demonstrated in numerous studies (17, 47). The method of PEGylation varies by manufacturer. Of the several PEG-FVIII molecules that have been created only three EHL products have reached clinical trials (Table III). An explanation for this phenomenon is the interference of PEG with the clotting activity of FVIII by altering its interaction with other molecules, including VWF (48). It has been hypothesized that PEGylation could have a beneficial impact on immunogenicity by interfering with the interaction of FVIII with antigen-presenting cells.

Rurioctocog alfa pegol (BAX-855, Adynovate) contains the native FL-rFVIII protein used in Advate coupled with 20-kDa branched PEG molecules (47). It was approved by the FDA in November 2015. Rurioctocog alfa pegol is prepared using a proprietary stable PEGylation technology developed by Nektar Therapeutics which is targeted at the

Table III. *Characteristics of extended half-life FVIII products.*

Product	Approval year	Modification	Cell line	Half-life (hours)	Prophylaxis regimen	Manufacturer	References
Efmoroctocog alfa (Eloctate)	2014	BDD-rFVIII with fused Fc	HEK	19	25-65 IU/kg every 3 to 5 days	Biogen/Sobi	(56-61)
Rurioctocog alfa pegol (Adynovate)	2015	Full-length rFVIII with 20-kDa nonspecific PEGylation	CHO	14-19.6	40-50 IU/kg 2 × weekly	Shire	(47, 49, 50)
Turoctocog alfa pegol (N8-GP)	-	B-domain truncated rFVIII with 40-kDa site-specific PEGylation	CHO	19	50 IU/kg every 4 days	Novo Nordisk	(48, 50, 53, 54)
Damoctocog alfa pegol (Bay-94-9027)	-	BDD-rFVIII with 60-kDa site-specific PEGylation	BHK	18.7	30-40 IU/kg twice weekly or 45-60 IU/kg every 5 days	Bayer	(51, 52)

rFVIII, recombinant factor VIII; Fc, fragment crystallizable; BDD, B-domain deleted; CHO, Chinese hamster ovary; HEK, human embryonic kidney; BHK, baby hamster kidney.

ε-amino groups of lysines (49). The manufacturers utilized techniques to remove the excess of free PEG reagent, as well as highly PEGylated and non-PEGylated rFVIII, and produced approximately 2 mol of PEG per FVIII molecule to enhance the PK profile of rFVIII (49). Two clinical studies were conducted in male patients with severe HA to assess the safety, efficacy, PK profile and immunogenicity of rurioctocog alfa pegol administered as prophylactic and on-demand treatments. Subjects on prophylaxis (40-50 IU/kg twice weekly) had a significantly reduced (90%) ABR compared with those following the on-demand treatment. The half-life of rurioctocog alfa pegol was found to be 1.4-fold longer (14-19.6 hours) than the half-life of Advate. No incidence of inhibitor development was described and no significant reagent-dependent differences between assays were seen (50).

Damoctocog alfa pegol (BAY-94-9027) is a B-domain truncated rFVIII variant that is site-specifically conjugated with a 60-kDa PEG linked via an amino acid residue substitution by cysteine. In a phase I trial, damoctocog alfa pegol was compared to rFVIII Kogenate FS and showed a half-life of about 19 hours (51). In the PROTECT VIII, phase II/III study, damoctocog alfa pegol showed a median ABR of 1.9 and 3.9 when given as prophylaxis every 5 (45-60 IU/kg) and 7 (60 IU/kg) days, respectively, in PTPs with severe HA (52). During the studies, the new EHL-FVIII was well tolerated without inhibitor development. In patients that receive damoctocog alfa pegol the FVIII:C can be measured using CSAs and OSAs that use an ellagic acid-based reagent (50).

Another glycoPEGylated agent, not yet approved for use, is turoctocog alfa pegol (N8-GP). Turoctocog alfa pegol corresponds to B-domain truncated rFVIII (turoctocog alfa) glycoPEGylated with a 40-kDa PEG that is attached via enzymatic transfer to a unique O-linked glycan in the 21-amino acid residual B-domain. It exhibited a mean half-life of 19.0 hours (range 11.6-27.3 hours), which represented approximately a 1.6-fold prolongation in comparison with rFVIII products previously used by the patients (53). With turoctocog alfa pegol the FVIII:C in OSAs varied with different activated partial thromboplastin time reagents leading to a significant underestimation (50). In clinical studies, turoctocog alfa pegol was in general well tolerated and a low-titer inhibitory anti-FVIII antibody was reported in only 1 patient after 93 days of exposure (54). The results of PATHFINDER 2, a phase III study in PTPs (adolescent and adult), showed a median ABR of 1.3 and 30.9 for prophylactic (50 IU/kg every 4 days) and on-demand treatment, respectively (53).

Fc fusion

The neonatal Fc receptor (FcRn) protects IgG and albumin from lysosomal breakdown and is involved in antigen presentation by professional antigen-presenting cells (44). The capacity of this receptor to extend the half-life of IgG and albumin has led to the development of new treatments (55). Fc fusion was the first half-life extension technology to be used in the hemophilia field. Fusion of the monomeric form of the IgG constant region (Fc) allows binding to the neonatal FcRn, delaying lysosomal degradation of Fc-containing proteins by cycling them back into circulation. The FVIII-Fc fusion protein induces half-life extension by altering the normal clearance (56).

Efmoroctocog alfa (rFVIIIFc, Eloctate) is a molecule in which Fc (from human IgG1) is linked to the C-terminus of BDD-rFVIII and is expressed in HEK293H cells (57). The phase III A-LONG study demonstrated an extended half-life of rFVIIIFc as well as its safety and hemostatic efficacy, including surgical prophylaxis, in previously treated adults/adolescents and children with severe HA (58-60). Results following tailored prophylaxis with efmoroctocog alfa (25-65 IU/kg every 3 to 5 days), according to individual patients' PK, revealed a median ABR of 1.6 (59).

Subsequently, interim data from the ASPIRE extension study confirmed the maintenance of low ABRs with extended prophylactic dosing intervals in patients receiving efmoroctocog alfa prophylaxis (61). The mean half-life of rFVIIIFc was found to be 19 hours and increased approximately 1.5-fold relative to that of standard rFVIII. This prolongation of half-life is a modest improvement compared to the 5-fold half-life extension achieved with rFIXFc (44). This limited improvement seems to be in part related to the close interaction of FVIII with VWF, its carrier protein in the circulatory system (62). It is possible to assess the plasma levels of FVIII activity after use of rFVIIIFc by CSAs and OSAs, although both can vary significantly by reagent (50). A phase III trial (ClinicalTrials.gov Identifier NCT02234323) in PUPs with severe HA is enrolling participants to evaluate the safety and efficacy of rFVIIIFc.

Recombinant porcine FVIII

The sequence differences between hFVIII and porcine FVIII (pFVIII) result in lower cross-reactivity of anti-hFVIII alloantibodies with pFVIII (63). For this reason pFVIII, derived from plasma, was used since the 1960s until 2004, when its manufacturing ceased due to concerns related to its safety and side effects (hypersensitivity and thrombocytopenia) (63). Susoctocog alfa (OBI-1, Obizur), a BDD recombinant pFVIII (r-pFVIII), has been produced in BHK cell lines and manufactured using two viral clearance steps to reduce the risk of potential pathogen transmission (64).

On the basis of the results of a prospective, multicenter, phase II/III study that evaluated the efficacy and safety of susoctocog alfa for the treatment of serious hemorrhagic events in patients with acquired HA (AHA), it was approved by the FDA as therapy for bleeds in patients with AHA. In the study, which included 28 patients with AHA, bleeds were successfully controlled with r-pFVIII in 86% (24/28) of subjects (64). Meanwhile, a phase II study assessing treatment of non-life/non-limb-threatening bleeding in patients with congenital HA and FVIII inhibitors showed that administration of eight or fewer injections of r-pFVIII resulted in hemostasis in 25 bleeding events (65). Patients tolerated r-pFVIII well without significant adverse events. A trial evaluating the use of r-pFVIII in patients with congenital HA and inhibitors undergoing surgery is currently ongoing (ClinicalTrials.gov Identifier NCT02895945).

Current Challenges and Perspectives

rFVIII was initially developed and produced in response to the need of a safe treatment, in terms of infection transmission, after the devastating sequelae of the AIDS and hepatitis epidemics of the 1980s in the hemophilia community. Since the risk that recombinant products can contain infectious contaminants is minimal, as there is a low probability that infectious agents may enter the cell culture and manufacturing processes, rFVIII products have largely eliminated concerns regarding pathogen transmission (21, 66).

Since 1988, the use of recombinant products has progressively increased and in many industrialized countries the plasma-derived products have nearly been completely replaced. Correspondingly, the worldwide demand of recombinant products raised up from 1.3 billion units in 1984 to 5.5 billion units

in 2008 (66). The growing availability and quality of recombinant coagulation factors have improved the quality of life of children with hemophilia and their families, have ensured home treatment and have contributed to the broad implementation of prophylactic treatment regimens in both adults and children (7, 66).

The major adverse consequence of using FVIII concentrates is the development of neutralizing inhibitors, which occur in about 30% of patients with severe HA and thus reduce or annul the response to treatment (67). The pathogenesis of inhibitory anti-FVIII antibodies is a multifactorial process that involves immune regulatory molecules, whose action and level are both genetically and nongenetically defined, and environmental factors. Nongenetic risk factors include those which are treatment-related, such as dosing regimen and type of product (68).

In the past, it was often suggested that there was a lower incidence of FVIII inhibitors with treatment based on plasma-derived concentrates than on recombinant concentrates. However, the studies performed to date have reported conflicting results (68), underlining the immunogenic role of infused FVIII, whether plasma-derived or recombinant. Only recently, the SIPPET study, a prospective randomized trial assessing the incidence of FVIII inhibitors, has reported a significantly increased risk of inhibitor development in PUPs treated with rFVIII compared with pdFVIII, raising the debate regarding factor concentrate-related immunogenicity (69).

Recently, rFVIII concentrates have been produced employing technological strategies to reduce FVIII immunogenicity (i.e., use of human cell lines, higher affinity towards VWF, no human or animal impurities) (36, 41). Moreover, results from preclinical studies support the notion that the Fc protein of rFVIIIFc has immunomodulatory properties (70, 71). However, the supposed lower immunogenic profile of these newer rFVIII concentrates needs confirmation in further studies including PUPs.

Even if the latest generations of rFVIII concentrates have led to some improvements, at the moment they have not eliminated the risk of inhibitor development. Therefore, in order to overcome the limitations of replacement therapy, there are emerging technologies that attempt to restore hemostasis outside FVIII/FIX replacement by using bioengineered substitutes that enhance thrombin generation or by inhibiting the natural anticoagulants (tissue factor pathway inhibitor, antithrombin and protein C) (72).

New nonfactor replacement therapies include a single molecule named emicizumab, which is a humanized, bispecific monoclonal antibody targeting FIXa and FX that can function as a FVIIIa mimetic. The bispecific monoclonal antibody emicizumab replaces only part of the full FVIIIa cofactor activity, and since it is a different protein, it differs greatly from FVIIIa in terms of affinity, regulation and typology, as well as regarding FIXa-enhancing activity (73).

Other nonfactor replacement therapies are i) concizumab, a humanized monoclonal IgG4 antibody that arrests the action of tissue factor pathway inhibitor; ii) fitusiran, a short-interfering RNA therapy that reduces antithrombin function in a dose-dependent way (74); and iii) activated protein C-specific serpin or protein S silencing RNA (75).

While concizumab and fitusiran are being evaluated in phase II/III trials, emicizumab has obtained FDA approval for the treatment of patients with HA and FVIII inhibitors (74). In fact, results from the HAVEN 1 and 2 studies showed that emicizumab prophylaxis was associated with a significantly lower rate of bleeding events in this setting of patients. It exhibits a long half-life (several weeks) and is administered via subcutaneous route, thus representing an attractive and significant change for hemophilia care (74).

These innovative strategies can be used for prophylaxis therapy, but some breakthrough bleeds are inevitable and these may require additional hemostatic treatment. The best choice of additional treatment in patients without inhibitors is represented by FVIII products, and by bypassing agents in the case of patients with inhibitors (76, 77). However, the need to treat breakthrough bleeding and avoid repeated high doses of bypassing agents requires the restoration of tolerance to FVIII in patients with inhibitors (76, 77).

The only proven effective therapy for inhibitor eradication is Immune Tolerance Induction (ITI), which involves repeated and persistent injections of replacement factor in order to downregulate the established antibody response and induce immune

tolerance. For the patients in whom ITI fails, options are few and are not clearly effective (72). Regarding type of concentrate, the choice of FVIII product to obtain inhibitor eradication is also a matter of debate (78). Even if there is no current information to suggest a more favorable outcome for one product over another, international consensus guidelines state that the majority of patients are tolerized with the same FVIII product that was in use at the time of inhibitor detection. This is a successful approach, and no evidence exists that supports switching to a different FVIII concentrate for de novo ITI. Otherwise FVIII concentrates containing VWF are currently recommended for salvage ITI in patients who have failed previous attempts using monoclonal or rFVIII products (79).

As summarized above, several different rFVIII concentrates, each with specific features, have been extensively validated in terms of hemostatic efficacy, immunogenicity and kinetic behavior, and are available for the treatment of patients with HA. However, in spite of these developments, replacement therapy with high-tech rFVIII remains problematic in many patients. Other technologies are currently being explored to allow, by additional modifications, subcutaneous administration of rFVIII and to significantly increase its half-life in blood. For instance, the PK, effectiveness and local distribution of subcutaneously administered turoctocog alfa pegol were recently evaluated in preclinical models; PK modeling predicted FVIII trough levels of 2.5-10% with turoctocog alfa pegol administered subcutaneously at a daily dose of 12.5 IU/kg^{-1} in 95% of patients with severe HA (80).

Conclusions

Although a cure for hemophilia has recently been achieved in some patients by gene therapy (81, 82), in the last 20 years the production of rFVIII has revolutionized the treatment of hemophilia in terms of security, efficacy and treatment burden. The availability of a wide choice of safe and increasingly effective concentrates, including newer rFVIII and EHL-FVIII products, has the potential to improve replacement therapy in the treatment of hemophilia. The novel therapeutic agents may overcome some of the remaining challenges in current HA treatment and offer the possibility of improving personalized/individual treatment with better outcomes. Therefore, although nonreplacement therapies and gene therapy appear to be very promising, rFVIII will very likely remain a critical component of the treatment of HA patients because of its physiological mode of action and unique ability to induce tolerance when inhibitors have developed. Even if the exposure to exogenous FVIII could be postponed or at least reduced in the future, it would appear totally premature to consider that replacement therapy with FVIII could be avoided.

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