



Recombinant Coagulation Factors and Thrombolytic Agents

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Koen Mertens and Alexander B. Meijer

Introduction

Blood coagulation and fibrinolysis are two sides of the same coin: the hemostatic system. Hemostasis involves the interplay of the coagulation cascade with activated blood platelets and the vessel wall. This results in the formation of a thrombus, which comprises activated platelets and cross-linked fibrin. Thrombus formation is counterbalanced by coagulation inhibitors on the one hand and the fibrinolytic system on the other hand. Upon vascular injury, coagulation (*thrombus formation*) serves to achieve local bleeding arrest, while fibrinolysis (*thrombus dissolution*) prevents obstruction of blood flow elsewhere in the circulation. Disruption of the balance between these mechanisms can cause malfunction of the hemostatic system and becomes manifest as bleeding or thrombosis. Bleeding disorders are usually due to a defect or deficiency in one of the constituents of the coagulation cascade and can be treated by substitution therapy to compensate for the missing component. Thrombotic disorders comprise two distinct forms: venous and arterial thrombosis. Venous thrombosis results from excessive coagulation and can ultimately lead to pulmonary embolism, while arterial thrombosis is associated with atherosclerosis, stroke, and acute coronary syndrome, the major indication for thrombolytic therapy. This chapter focuses on the recombinant biopharmaceuticals that are currently available for the treatment of bleeding and thrombosis.

K. Mertens (✉)

Department of Pharmaceutical Sciences, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands
e-mail: k.mertens@uu.nl

A. B. Meijer

Department of Molecular Hematology, Sanquin Research, Amsterdam, The Netherlands
e-mail: s.meijer@sanquin.nl

The Essentials of Blood Coagulation and Fibrinolysis

Formation and Dissolution of the Hemostatic Plug

While the network of hemostasis and thrombosis is generally perceived as being a complex issue in physiology, understanding the essentials thereof will greatly facilitate appreciation of the biopharmaceuticals used in this area, and in particular, of the pharmacodynamics thereof. As schematically depicted in Fig. 18.1, the system can be divided into three distinct sections. The left part (panel a) comprises the coagulation cascade. This is an ordered conversion of proenzymes into active enzymes that leads to the activation of prothrombin into thrombin. Once the initial amounts of thrombin are formed, it catalyzes a variety of enzymatic conversions. These include a self-amplifying loop by feedback activation of several other components upstream in the cascade and self-dampening of its own formation by activating an enzyme that specifically inactivates essential cofactors in the cascade. The central part (panel b) summarizes how thrombin drives the formation of the hemostatic plug, initially by activating platelets and converting fibrinogen into fibrin polymers, and subsequently by activating factor XIII, which then cross-links fibrin into a stable network. The right section (panel c) represents the fibrinolytic system, in which activators like tissue-type plasminogen activator (t-PA) convert plasminogen into the enzyme plasmin that degrades cross-linked fibrin into soluble fragments, resulting in lysis of the hemostatic plug.

The Network of Coagulation Factors

Why do we need so many coagulation factors? The answer lies in the various regulatory steps, which allow the mechanism to act as a biological amplifier that rapidly responds to injury, while at the same time the process should remain restricted to the site of injury. The apparent complexity of the coagulation scheme in Fig. 15.1 (panel a) merits some further explanation. Apart from a few exceptions, the proteins in

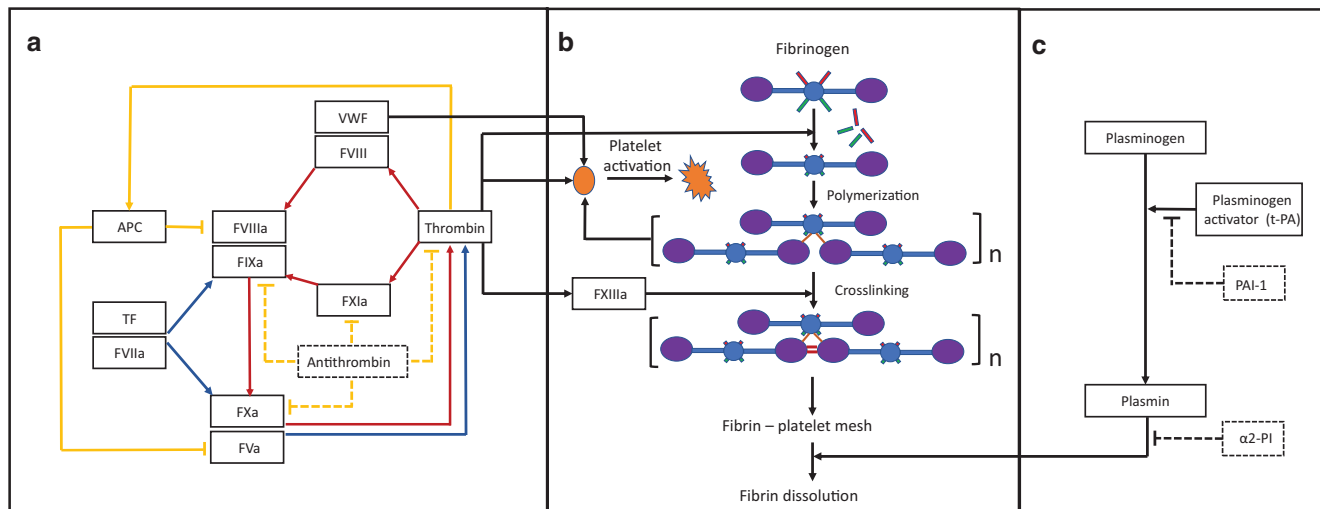


Fig. 18.1 Schematic representation of hemostasis. Panel (a) the coagulation cascade; Panel (b): the hemostatic plug; Panel (c): the fibrinolytic system. The various phases in the coagulation cascade comprise initiation (blue), propagation (red), and termination (yellow). The constituents thereof are further described in the text. Roman numerals refer to individual coagulation factors. Von Willebrand factor is abbreviated as *VWF*, *APC* activated protein C, *TF* tissue factor, *PAI-1* plasminogen activator inhibitor-1, $\alpha 2$ -*PI* $\alpha 2$ -plasmin inhibitor

this system ('factors') are indicated by roman numerals, with the suffix 'a' referring to their activated form. This nomenclature dates from 1959 and has been implemented to resolve confusion by the coexistence of multiple names for the same component. For some factors, however, the old names have remained in use, such as fibrinogen (factor I), prothrombin (factor II), and Tissue Factor (factor III). The terms factor IV and factor VI are obsolete, as they have never been assigned to a specific protein. Of the other coagulation factors, numbers V and VIII are cofactors, while the remaining numbers VII and IX to XII are proenzymes of serine proteases that catalyze the sequential steps in the cascade (Furie and Furie 1988).

The concept of an ordered sequence of reactions as a cascade or waterfall has first been described in 1964 (Davie and Ratnoff 1964; Macfarlane 1964) and has remained essentially unaltered since then. What did change, however, is the insight into the various feedback effects of thrombin upstream in the cascade. This introduced a distinction between successive stages in thrombin formation, which are often referred to as *initiation*, *propagation*, and *termination* (Mann et al. 2009). In each of these phases, different sections of the cascade predominate. These can be summarized as follows:

1. In the *initiation phase*, indicated by blue arrows in Fig. 18.1a, vascular injury leads to the exposure of tissue factor (TF). The cascade then runs from the FVIIa/TF to FXa/FVa complex to the conversion of prothrombin into thrombin. The initially formed thrombin starts activating platelets and cleaving fibrinogen into fibrin (Fig. 18.1b), and at the same time, initiates the self-amplifying propagation loop.

2. This *propagation phase*, indicated by red arrows, involves the activation of factor XI (FXI) and the cofactors factor VIII (FVIII) and factor V (FV). The cascade then runs from the FXIa (or FVIIa) to FIXa/FVIIIa complex and further from the FXa/FVa complex to generate large amounts of thrombin. The main difference between the initiation and the propagation phase is that the latter comprises an additional amplifying step, which involves FVIII and FIX.
3. Finally, in the *termination phase*, indicated by the yellow arrows, thrombin binds to an endothelial receptor (thrombomodulin) and activates Protein C. Once activated, this inactivates the cofactors FVIIIa and FVa and thereby causes thrombin formation to shut-down. The activated coagulation enzymes are neutralized by antithrombin (yellow dashed lines).

Local Control Mechanisms

How do the various mechanisms shown in Fig. 18.1 remain localized to the site of injury? One reason is that assembly of enzyme-cofactor complexes in the coagulation cascade requires membranes containing acidic phospholipids, which are exposed on perturbed cells such as activated platelets, but not on resting cells. Moreover, any excess of activated factors is neutralized by protease inhibitors that occur in plasma. These include tissue factor pathway inhibitor, which inhibits at the level of factor VIIa, and antithrombin, an inhibitor of most of the other enzymes from the cascade. Similar considerations apply to the fibrinolytic system (Fig. 18.1c). First, most t-PA is stored in vascular cells and released upon perturbation, while levels of t-PA in circulation are low. Second, plasmin needs its substrate, fibrin, to express full fibrinolytic

activity. Finally, the circulating inhibitors plasminogen activator inhibitor-1 (PAI-1) and α 2-plasmin inhibitor (α 2-PI) neutralize the excess of their target proteases (indicated by dashed lines in Fig. 18.1c).

Hemostatic Disorders

There is little or no redundancy within the intricate protein network involved in the formation and dissolution of the hemostatic plug. Therefore, deficiency or dysfunction of a single component may have a severe impact. The consequences thereof can include both bleeding and thrombosis. Deficiency of most of the coagulation factors results in a bleeding tendency, while a shortage of coagulation inhibitors such as antithrombin or protein C predisposes to thrombosis. Likewise, thrombosis may also result from a defect in the fibrinolytic pathway, while bleeding has been associated with a deficiency in α 2-plasmin inhibitor. The incidence of deficiencies in hemostatic proteins is summarized in Table 18.1 (adapted from Bishop and Lawson 2004 and Palla et al. 2015). In affected patients, bleeding can be treated by administering the missing plasma protein. This may be of human origin, as a concentrate derived from human plasma, or a biopharmaceutical from recombinant sources. Table 18.1 lists the availability of such protein therapeutics. It is evident that, with a few exceptions, recombinant products have been clustering around the field of hemophilia, which due to its relatively high incidence, represents the most significant market.

Hemophilia and Other Bleeding Disorders

Hemophilia is the best-known bleeding disorder, as it occurred in several European royal families, including the Romanov dynasty in Russia. These descendants of the British Queen Victoria had a defect in the gene encoding in

factor IX and thus suffered from hemophilia B (Rogaev et al. 2009). The more frequent disorder, however, is hemophilia A, or factor VIII deficiency. Hemophilia A and B are clinically indistinguishable, which is to be expected because factor VIII and factor IX participate in the same step in the coagulation cascade: the activation of factor X. Lack of either the cofactor or the enzyme moiety in the FVIIIa/FIXa complex disrupts the biological amplifier that drives the propagation phase in thrombin generation (see Fig. 18.1). Hemophilia differs from the other inherited bleeding disorders in that the genes encoding factor VIII and factor IX are located on the X-chromosome. The inheritance pattern is X-linked recessive, and mutation in the single X-chromosome in males is sufficient to cause the deficiency. In females, however, the same mutation in one X-chromosome results in heterozygosity and in factor levels which, although reduced, still are sufficient to support normal hemostasis.

The other coagulation factor deficiencies do not display this sex-linked pattern, and a complete deficiency thereof will need a defect in both chromosomes (homozygous or compound heterozygous). For most of these deficiencies, often referred to as the Rare Bleeding Disorders, substitution therapy is available, albeit to a limited extent, in the form of concentrates from human blood (see Table 18.1).

The most common bleeding disorder is von Willebrand's disease. This is caused by a deficiency or dysfunction of the von Willebrand factor, a large multimeric adhesion protein that mediates the interaction between activated platelets and the perturbed vessel wall at the site of injury. As such, the von Willebrand factor is an essential constituent of the hemostatic plug (see Fig. 18.1b). Besides its role as an adhesion protein, the von Willebrand factor serves as carrier of factor VIII in the circulation and protects factor VIII from degradation and premature clearance. Within von Willebrand's disease, a variety of subtypes can be distinguished, with partial quantitative deficiency and different molecular defects (for

Table 18.1 Deficiency of hemostatic proteins: incidence and options for substitution therapy

Deficiency	Incidence	Bleeding phenotype	Availability of products for substitution therapy	
			Human plasma-derived	Recombinant
Fibrinogen (factor I)	1 in 1 million	None to severe	Few	None
Prothrombin (factor II)	1 in 2 million	Mild to moderate	Common	None
Factor V	1 in 1 million	Moderate	One (under evaluation)	None
Factor VII	1 in 500,000	Mild to severe	Few	Factor VIIa only
Factor VIII (hemophilia A)	1 in 5000 males	Moderate to severe	Common	Several (see Table 18.2)
Factor IX (hemophilia B)	1 in 25,000 males	Moderate to severe	Common	Several (see Table 18.3)
Factor X	1 in 1 million	Mild to severe	Common	None
Factor XI	1 in 1 million	Mild to moderate	Few	None
Factor XIII	1 in 2 million	Moderate to severe	Few	One
von Willebrand factor	~1 in 1000	Mild to severe	Common	One
α 2-plasmin inhibitor	very rare	Mild to severe	None	None
Antithrombin	~1 in 5000	Thrombotic phenotype	Common	One

review, see Castaman and Linari 2017). Only the most severe deficiency (type III, 1–2% of all cases) requires substitution therapy with von Willebrand factor-containing therapeutics.

Thrombosis

While protein substitution therapy is a straightforward approach to stopping or preventing bleeding, the management of thrombosis is more complex. Partial deficiencies of antithrombin or protein C are risk factors that predispose to venous thrombosis, but usually multiple, often acquired risk factors, are involved. The mechanisms involved in thrombosis are beyond the scope of the present chapter and have been reviewed elsewhere (Mackman 2008). Treatment involves dampening the coagulation cascade or inhibiting platelet activation, usually by small molecules rather than protein therapeutics. Protein substitution therapy remains limited to some specific cases of antithrombin deficiency.

As for arterial thrombosis, this is associated with atherosclerosis. After the rupture of an atherosclerotic plaque, a platelet-rich thrombus may lead to arterial occlusion, which then may result in ischemia, myocardial infarction, or stroke (Lusis 2000; Mackman 2008). In such situations, lysis of the occluding thrombus is the immediate target. This can be accomplished by infusing large amounts of plasminogen activators. The medical need for such thrombolytic therapy is high. This explains why tissue-type plasminogen activator, after human insulin and human growth hormone, was among the first recombinant protein therapeutics that reached the market.

Recombinant Factor VIII

In the early 1980s, the factor VIII cDNA has been cloned by two independent groups, one collaborating with Genentech (Vehar et al. 1984), and another with Genetics Institute (Toole et al. 1984). This provided access to the biotechnological development of recombinant factor VIII. Factor VIII was among the largest proteins characterized at that time. The factor VIII gene spans 180 kb and comprises 26 exons that together encode a polypeptide of 2351 amino acids. This includes a signal peptide of 19 amino acids and a mature protein of 2332 amino acids. As reviewed in detail elsewhere (Lenting et al. 1998; Fay 2004), factor VIII displays the ordered domain structure A1-*a1*-A2-*a2*-B-*a3*-A3-C1-C2 (see Fig. 18.2a). The triplicated A-domains share homology with the copper-binding protein ceruloplasmin, while the two C-domains are structurally related to lipid-binding proteins such as lactadherin. The A-domains are bordered by short spacers (*a1*, *a2*, and *a3*) that are also known as acidic regions. These spacers contain sulfated tyrosine residues that are needed for full biological activity.

The central B-domain carries the vast majority of the glycosylation sites in factor VIII. Due to processing at the junction B-*a3*, mature factor VIII is secreted as a heterodimer of the heavy chain (A1-*a1*-A2-*a2*-B) and light chain (*a3*-A3-C1-C2). In the circulation, the B-domain is subject to cleavage at multiple sites, resulting in size heterogeneity without apparent consequence for biological activity (Andersson et al. 1986). Thus, about one-third of the factor VIII protein seems dispensable for biological activity. Indeed, recombinant expression of a factor VIII variant lacking a major part of the B-domain proved functionally normal (Toole et al. 1986). For B-domain deleted factor VIII, several crystal structures are available. These reveal a compact cluster of the three A-domains, supported by a tandem of the two C-domains, in a side-by-side orientation (Fig. 18.2b). This structure represents the inactive factor VIII, and conversion into factor VIIIa involves cleavage at the *a1*-A2, *a2*-B, and *a3*-A3 junctions. The resulting heterotrimer is the molecular form that assembles with factor IXa to activate factor X in the coagulation cascade.

Factor VIII Products

Due to the size and complexity of the factor VIII protein, mammalian cells need to be used as an expression system. Commonly used cells are CHO (Chinese hamster ovary), BHK (baby hamster kidney), and HEK (human embryonic kidney) or engineered derivatives thereof. Post-translational modifications (see Fig. 18.2a) include glycosylation and the sulfation of specific tyrosine residues in the *a1*, *a2*, and *a3* segments. One particular challenge is its low expression, as factor VIII tends to accumulate intracellularly, which is cytotoxic for the host cells. Another challenge is the instability of factor VIII once secreted. Over the past 40 years, these issues have been resolved by a limited number of manufacturers only. An overview of the various products is given in Table 18.2.

Full-Length Factor VIII

The first generation of recombinant factor VIII was intended to provide exact copies of the natural protein from human plasma, which had been established as being effective in the treatment of hemophilia A since the 1960s. The first products, Kogenate (developed at Genentech and Bayer) and Recombinate (developed at Genetics Institute and Baxter) were licensed in 1992. In order to enhance secretion and stability, Recombinate was produced by co-expression with the von Willebrand factor, which was removed during downstream processing. The simultaneous expression of these two exceptionally large polypeptides was a remarkable achievement in the early years of biopharmaceuticals.

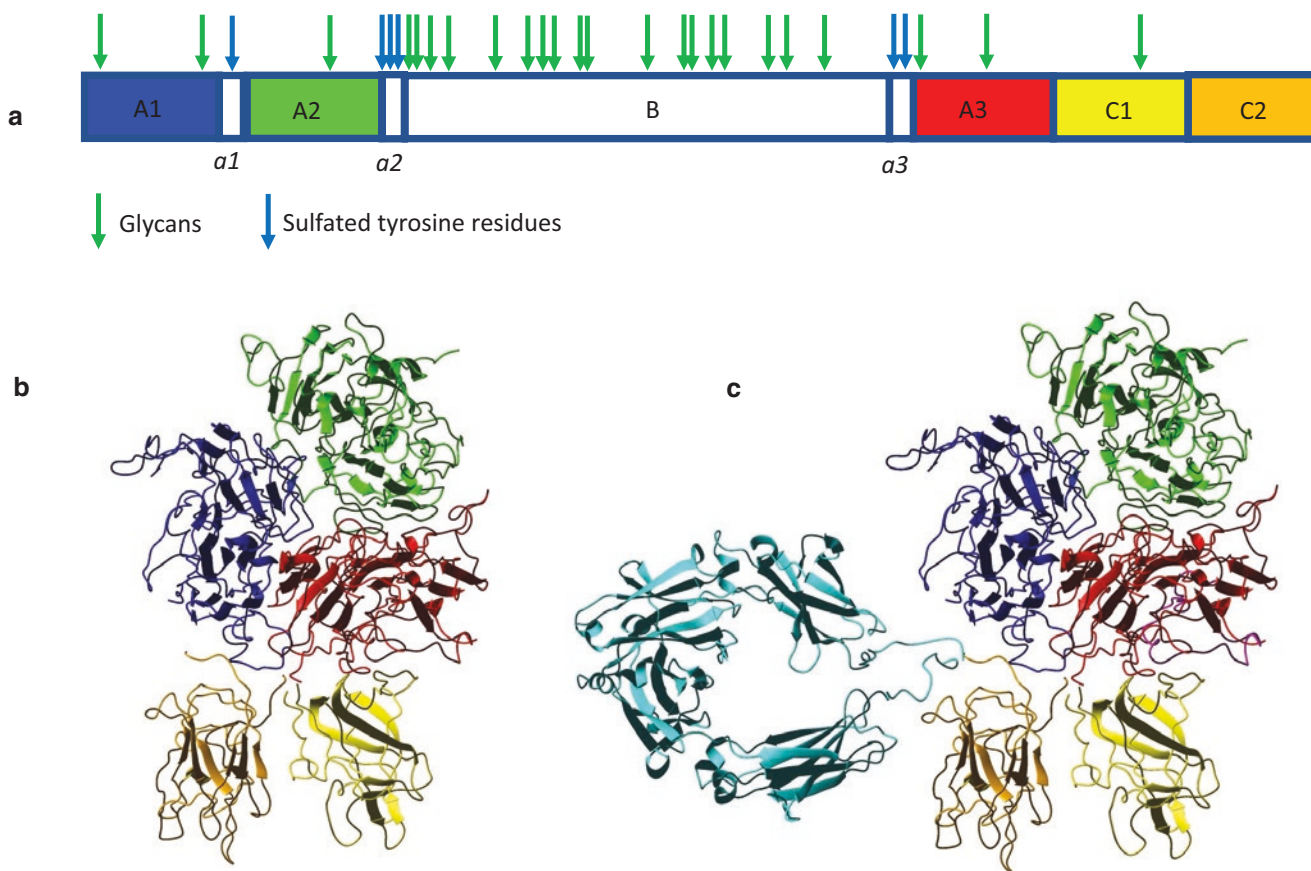


Fig. 18.2 Structure of factor VIII. Panel (a) displays the domain structure of full-length factor VIII. Panel (b) represents the 3D-structure of B-domain-deleted factor VIII (derived from pdb 2R7E), and panel (c) is the same structure with the Fc fragment of IgG1 (from pdb 1HZA) fused to the C-terminus of the C2-domain

Both Kogenate and Recombinate were produced using the human albumin-containing culture medium and employed an albumin-containing formulation for the final product. As such, these initial products were actually plasma-derived, except that the active ingredient was produced through recombinant technology. In 2000, Kogenate has been replaced by Kogenate-FS, with an albumin-free formulation. In 2016, this was followed by Kovaltry, which employs co-expression with heat shock protein-70 to facilitate factor VIII expression in protein-free medium (Table 18.2). As for Recombinate, a protein-free equivalent (Advate) became available in 2003. A PEGylated variant thereof, Adynovate, was licensed in 2015 (see below, section “Extended Half-Life Factor VIII”).

B-Domain Deleted (BDD) Factor VIII

The finding that the central B-domain is dispensable for *in vitro* activity (Toole et al. 1986) formed the basis for the design of a variety of deletion mutants. An advantage of B-domain deletion is that this greatly improves factor VIII expression. The first BDD-factor VIII was ReFacto

(developed at Pharmacia/Pfizer), which was followed in 2009 by the protein-free equivalent ReFacto-AF/Xyntha. Other BDD-factor VIII products have slightly different deletions (see Table 18.2). All of them have conserved a small N-terminal part of the B-domain, in order to maintain the thrombin cleavage site at the *a2*-B junction (position 740–741). Moreover, most constructs have retained the processing site at the B-*a3* junction (position 1648–1649) and consequently are, like wild-type factor VIII, heterodimeric molecules (NovoEight, Nuwiq). An exception is Afstyla, which is a single-chain molecule because the deletion extends into the *a3* segment. Despite the differences between the individual constructs, these BDD-factor VIII products, once activated, are fully indistinguishable from wild-type factor VIIIa, because the B-domain remnant and the *a3*-section are released upon activation. Other BDD factor VIII products, in particular those that have been engineered to extend half-life (see Table 18.2), may still contain the introduced modification within their activated form (see following section).

Table 18.2 Recombinant factor VIII products

Product type	INN name	(Investigational) product name(s)	First approval (USA/EU)	Half-life (mean, h)	Production technology	Reference
Full-length FVIII	octocog alfa	Recombinate	1992	12	CHO cells, co-expression with VWF, albumin in culture and formulation	
		Advate	2003	10-14	as Recombinate, but without protein in culture, albumin-free formulation	
		Kogenate Helixate	1992	14-17	BHK cells, albumin in culture and formulation	
		Kogenate-FS	2000	14-17	as Kogenate, but with albumin-free formulation	
B-Domain Deleted FVIII	rurioctocog alfa pegol	Kovaltry, Iblis (<i>BAY 81-8973</i>)	2016	13	BHK, co-expression with HSP-70, protein-free culture and formulation,	Maas Enriquez et al. (2016)
		Adynovate (<i>BAX 855</i>)	2015	14-16	as Advate, 20kDa pegylation to extend half-life	Turecek et al. (2012)
	morococog alfa	ReFacto	1999	14	CHO cells, deletion of amino acids 744-1637, albumin in culture, not in formulation	
		ReFacto-AF Xyntha	2008	13	as ReFacto, albumin-free culture and formulation	
	turoctocog alfa	NovoEight (<i>N8</i>)	2013	11	CHO cells, deletion of amino acids 751-1637, albumin-free culture and formulation	Thim et al. (2010)
	simococog alfa	Nuwiq	2014	17	HEK cells, B-domain replaced by 16 amino acid linker, protein-free culture and formulation	Sandberg et al. (2012)
	lonococog alfa	Afstyla (<i>rVIII-SingleChain</i>)	2016	14	CHO cells, deletion of amino acids 765-1652, single-chain molecule, albumin-free	Schmidbauer et al. (2015)
	efmorococog alfa	Eloctate, Elocta (<i>rFVIII-Fc</i>)	2014	20	HEK cells, deletion of amino acids 744-1637, dimeric IgG1-Fc fused to FVIII C2-domain	Leksa et al. (2017)
	damococog alfa pegol	Jivi (<i>BAY 94-9027</i>)	2018	~19	HEK-hybrid cells, deletion of amino acids 743-1636, Lys-1804 substituted by Cys for site-specific 40kDa pegylation in the A3-domain	Mei et al. (2010)
	turoctocog alfa pegol	Esperoct (<i>N8-GP</i>)	2019	~19	as NovoEight, with site-specific glycopegylation in residual B-domain segment	Stennicke et al. (2013)
efanesococog alfa	Altuviio (<i>BIVV001</i>)	2023	~41	dimeric IgG1-Fc fusion as in Eloctate, with added fusion with FVIII-binding region of VWF and two half-life extending (XTEN) insertions	Lissitchkov et al. (2022)	
FVIII mimic	Emicizumab	Hemlibra (<i>ACE910</i>)	2018	4-5 weeks	Recombinant humanized bispecific antibody against FIXa and FX for subcutaneous administration	Uchida et al. (2016)

Extended Half-Life Factor VIII

One limitation of factor VIII replacement therapy is the relatively short half-life, which may vary between 10 and 17 h (see Table 18.2). Half-life extension would provide an obvious therapeutic advancement. Over the past few years, several products have been developed to pursue this goal. Thus, the original paradigm that recombinant factor VIII should be identical to the natural protein has started shifting toward engineering to improve on nature.

Adynovate is a PEG (polyethylene glycol)-ylated full-length factor VIII, based on Advate. It contains on average 2 moles of branched 20 kDa PEG per factor VIII molecule.

Approximately 60% of the PEG chains are located in the B-domain and thus are lost upon activation. The remaining modifications are randomly dispersed over the rest of the protein and, by virtue of the low extent of PEGylation, do not interfere with any known functional interaction (Turecek et al. 2012).

Eloctate is a fusion between BDD-factor VIII and the Fc fragment of IgG. This serves to target factor VIII to the FcRn receptor recycling pathway that endows IgG and albumin with their long half-life (see Chap. 8). The fusion of the Fc fragment to the factor VIII C-terminus yields a derivative in which the activated form is non-natural in that it still carries

the Fc moiety. Surprisingly, this C-terminal extension conserves factor VIII's potency, presumably because the Fc portion is loosely attached and, besides the linker sequence, has no specific interaction with the factor VIII surface (Leksa et al. 2017). On the other hand, the fusion protein displays an aberrant response in some activity assays used for therapy monitoring (see section "Clinical Considerations").

Another product in this category is Esperoct. This is a PEGylated version of NovoEight (N8-GP, see Table 18.2). This is modified with a branched 40 kDa PEG using enzymatic glycoconjugation, which is targeted to the single O-linked glycan in the short B-domain remnant. Because the PEG moiety is released upon activation, the active form is the natural, unmodified factor VIIIa. A different strategy has been used for the recently approved Jivi (BAY 94–9027, see Table 18.2), developed by Bayer. This employs cysteine-targeted PEGylation with a branched 60 kDa PEG. This cysteine is introduced by mutagenesis in the factor VIII A3 domain. The PEG moiety remains conserved after activation, and thus the active species in this product is non-natural. This has some impact on activity assays used for monitoring (see section "Clinical Considerations").

The benefit of these half-life extensions seems limited (Table 18.2). For the current four products, cross-over studies reported a 1.5- to 1.7-fold half-life prolongation over an unmodified comparator (Peyvandi et al. 2013). So far, this seems to be the maximum achievable prolongation by such modification strategies. This limitation is because factor VIII, whether or not modified to extend its half-life, circulates in complex with the recipient's endogenous von Willebrand factor. Therefore, the clearance of the von Willebrand factor remains the major driver of factor VIII's half-life (Pipe et al. 2016). To overcome this limitation, a novel engineered factor VIII variant (BIVV001, Efanesoctocog alfa) has been designed in which factor VIII is fused with a fragment of the von Willebrand factor comprising the factor VIII-binding region. This fusion construct further comprises a dimeric Fc fragment and two unstructured, so-called 'XTEN' insertions, together resulting in a 3 to four-fold half-life extension (see Table 18.2).

Factor VIII Mimics

A remarkably innovative approach is to design proteins that are totally unrelated to factor VIII but share its biological activity. This is based on the notion that factor VIIIa assembles with FIXa, and facilitates the proper alignment with factor X that is needed for full activity in the propagation phase of coagulation (see Fig. 18.1). This has been accomplished by creating a humanized bispecific monoclonal antibody, wherein one IgG-arm binds to factor IXa, and the other to factor X. This compound, initially called ACE910, now Emicizumab, was developed at Chugai Pharmaceutical as a treatment for hemophilia A patients with inhibitors against

FVIII but finally has been licensed for use in hemophiliacs both with and without inhibitors (Hemlibra, Table 18.2). A major advantage of an antibody over factor VIII is that it has a much longer half-life and allows for subcutaneous administration. (see Chap. 8 for details on monoclonal antibodies). Another difference is that factor VIII circulates in an inactive form, while the antibody is not under such proteolytic control. This carries the inherent risk of causing thrombotic events. For Hemlibra, safe dosage regimens for prophylaxis have been established between 1.5 mg/kg once weekly and 6 mg/kg every 4 weeks. As such, Hemlibra provides a valuable innovation in the repertoire of factor VIII-based therapeutics.

A novel factor VIII mimetic antibody, designated Mim8, has been developed at Novo Nordisk (Østergaard et al. 2021). One difference with Hemlibra is that the factor IXa-binding arm has been specifically optimized to also enhance the proteolytic activity of factor IXa. Preclinical data suggest that Mim8 is significantly more potent than Hemlibra. Whether or not this translates into a clinical benefit is currently under investigation.

Pharmacology

The term factor VIII, or Anti-Hemophilic Factor in the early US literature, has been introduced before the protein had been identified. Factor VIII was defined by its function as the activity that corrects the clotting defect of hemophilic plasma. This functional definition has remained the basis for the quantification of factor VIII since then, and factor VIII concentrations are expressed in units, where 1 unit represents the amount of factor VIII activity in 1 mL of normal human plasma. Besides in units/mL, factor VIII levels in plasma are often also expressed as % of normal. Thus, the assessment of product potency, dosing, and pharmacokinetics is based on bioassays, with their inherent variability.

Pharmacokinetics

The pharmacokinetics of factor VIII is largely dependent on the plasma level of the von Willebrand factor, which stabilizes factor VIII and protects against premature clearance. As reviewed in detail elsewhere (Björkman and Berntorp 2001), systemic clearance in patients with normal von Willebrand factor levels is typically 3 mL/h/kg, with an apparent distribution volume that slightly exceeds the patient's plasma volume, and an average elimination half-life of approximately 14 h. While assessment of pharmacokinetics is mandatory for all individual factor VIII products, there is quite some variability between the data reported (see Iorio 2017 and Table 18.2). Apart from variation between patients, technical issues contribute to this variation, such as the sampling time points, and the dependence on bioassays for the determina-

tion of the administered dose and plasma levels post-infusion. Given this variability, the pharmacokinetics of the various recombinant factor VIII products can be considered as being essentially similar, with the exception of those products that have been engineered to extend half-life.

Pharmacodynamics

Hemophilia A is a congenital deficiency or dysfunction of factor VIII which, dependent on the residual level of factor VIII activity, is categorized as mild (6–40%), moderate (1–5%), or severe (<1% of normal) (for review, see Franchini and Mannucci 2013). Administration of factor VIII serves as a substitution therapy, with the objective of restoring the defect in thrombin generation. Normalization of factor VIII levels in the circulation requires administration by the intravenous route. The therapeutic range is rather narrow, because supra-normal factor VIII levels increase the risk of thrombosis, while the risk of bleeding tends to increase when levels drop below 40%. A usual strategy is to maintain a dosing schedule that ensures that factor VIII trough levels remain at least at 1–4% of normal to avoid the bleeding risk of the severe form of hemophilia A.

For all licensed factor VIII products, efficacy has been established for both on-demand and prophylactic treatment. Dosing is generally based on the empirical finding that 1 unit factor VIII per kg body weight raises the plasma factor VIII activity by 2%, which implies the simple formula (Björkman and Berntorp 2001):

$$\text{dose (units / kg)} = 50 \times (\text{required rise in units / mL factor VIII})$$

On-demand treatment involves factor VIII administration once bleeding occurs. Dependent on the type of bleeding, this requires administration of 20–100 units/kg, which can be repeated every 8–24 h if needed (Franchini and Mannucci 2013). In developed countries, the general treatment has the objective not to treat but rather to prevent bleeding episodes. This can be short-term prophylaxis to provide protection during surgical or invasive procedures or long-term prophylaxis to prevent spontaneous bleeding. The latter involves regular treatment at a dose of 20–40 units/kg, which needs to be repeated 2–3 times per week in order to maintain the factor VIII level above 1% of normal. For extended half-life products, a recommended regimen is 50 units/kg every 4–5 days (Cafuir and Kempton 2017).

For all factor VIII products, the main side effect is the formation of antibodies against factor VIII (inhibitors) once replacement therapy is started. This involves 30–40% of all severe patients. In the majority of cases, these antibodies are transient and can be eliminated by immuno-tolerance therapy with high doses of factor VIII. In a significant proportion of the patient population, however, the antibodies remain

persistent and thereby preclude further treatment with factor VIII (Franchini and Mannucci 2013). In those cases, bleeding can be controlled using factor VIII-bypassing agents, including recombinant factor VIIa (see below) and bispecific antibody factor VIII mimics.

Pharmaceutical Considerations

While the factor VIII concentration in human plasma is approximately 0.1 µg/mL, the potency of factor VIII products is expressed in International Units (IU). This is an activity unit relative to a reference preparation that is established from time to time by the World Health Organization. Products are available in fillings ranging between 250 and 2000 (or 3000) IU in order to provide convenient dosing for children and adults using a single vial. Current products are freeze-dried and need to be reconstituted before infusion. Most products are stabilized in a sucrose-containing formulation and have a shelf life of 24–36 months upon storage at 2–8 °C. For most products, data are available that establish stability at room temperature for 6–12 months, thus facilitating patients to travel while maintaining their prophylactic treatment.

As for impurities, most products may contain low amounts of residual proteins from the host expression system. With the exception of Nuwiq, which is expressed in a human cell line, these products may cause side effects in patients with hypersensitivity against rodent proteins. This adverse event, however, remains rare. To further enhance safety, the downstream processing of most recombinant factor VIII products includes virus-eliminating steps that have been previously established for plasma-derived factor VIII products, such as immuno-affinity chromatography, solvent/detergent treatment, ultrafiltration, and combinations thereof (Franchini and Mannucci 2013).

Clinical Considerations

The sole indication for recombinant factor VIII is congenital hemophilia A that is not complicated by factor VIII inhibitors. Similarly, acquired hemophilia A, which is due to the spontaneous formation of anti-factor VIII antibodies should be treated using alternative therapies such as factor VIII bypassing agents.

For all recombinant factor VIII products, appropriate treatment protocols have been established as part of the licensing procedure. Nevertheless, there is an increasing trend toward personalized prophylactic treatment based on the pharmacokinetic profile of individual patient/product combinations. The advantage of this policy is that it allows the precise prediction of trough values and adaptation of

dosing schedules accordingly. As for the extended half-life products, these tend to be used not to increase the dosing intervals but rather to maintain higher trough levels and thus to offer better protection against spontaneous bleeding (Iorio 2017). A crucial requirement for this approach to be allowable is the availability of robust methods for monitoring post-infusion factor VIII levels. While this is feasible for full-length products, this remains a problematic issue for many of the engineered factor VIII products, including some products with B-domain deletions, the single-chain factor VIII product, some of the PEGylated products, and the Fc-fusion protein. While some manufacturers recommend the use of specific assay reagents, or reagent-dependent conversion factors, it seems evident that this approach lacks robustness, and as such is complicating the personalized use of these products (Kitchen et al. 2017).

Recombinant Factor IX

While the cDNA encoding factor IX has been cloned in 1982 (Choo et al. 1982; Kurachi and Davie 1982), it took until 1997 before the first recombinant factor IX was licensed. Expressing the protein proved challenging in that factor IX requires excessive post-translational modification for full biological activity. These include 12 glutamine residues in the N-terminal section of the mature protein, which need to be modified into γ -carboxyglutamic acid (conversion of Glu into Gla) by vitamin K-dependent carboxylase. The modification involves interaction between carboxylase and the 46 amino acid long propeptide of factor IX, which is cleaved off by a furin-like processing enzyme prior to secretion (Jorgensen et al. 1987; Wasley et al. 1993). This results in a mature protein of 416 amino acids with the Gla-rich segment (the Gla-domain) at its N-terminus. Factor IX further comprises two epidermal growth factor-like (EGF) domains, an activation peptide (AP), and the catalytic domain (see Fig. 18.3a). Cleavage at both sides of the activation peptide yields a two-chain protein, consisting of a heavy chain (the catalytic domain) and a light chain (the Gla-EGF1-EGF2 section). Structural data indicate that factor IX is a stretched molecule, with the catalytic domain on one end and the Gla-domain, with its Gla-residues protruding, at the opposite end (Perera et al. 2001, see Fig. 18.3c).

Why are these Gla residues so important? Like in other vitamin K-dependent coagulation factors, such as factor VII, factor X, protein C, and prothrombin, they provide high-affinity binding sites for Ca^{2+} -ions and thereby mediate the interaction with negatively charged lipid membranes. Thus, the appropriate assembly of coagulation factors at the site of injury is fully dependent on the presence of these clustered

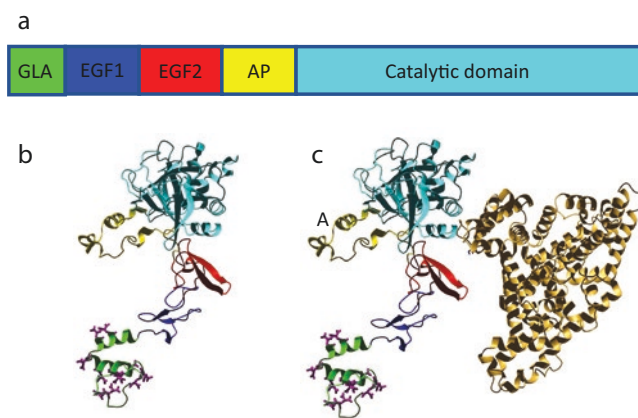


Fig. 18.3 Structure of factor IX. Panel (a) displays the domain structure. The colors of the individual domains correspond to those in panel (b), which provides a 3D structure of factor IX (Perera et al. 2001). In the Gla-domain, the γ -carboxylated residues are indicated in purple. Panel (c) provides the same structure with human albumin (from pdb 1AO6) fused to the C-terminus of the catalytic domain. EGF epidermal growth factor, AP activation peptide

Ca^{2+} -binding sites (Furie and Furie 1988). Therefore, full carboxylation is a major requirement in the production of recombinant factor IX.

Factor IX Products

The complexity of the post-translational modifications remains a major challenge in the production of recombinant factor IX. Mammalian cells need to be used as an expression system, because these contain the necessary carboxylase enzyme complex. However, the capacity of host cells to perform this post-translational modification tends to be limiting, leading to under-carboxylated forms that lack full activity. Biological activity further requires the removal of the propeptide, which usually implies the need for co-expression with a furin-like processing enzyme (Harrison et al. 1998). Most other post-translation modifications are located in the activation peptide and include two N-linked glycosylation sites, tyrosine sulfation, and serine phosphorylation. With respect to these modifications, recombinant factor IX remains non-identical to its plasma-derived counterpart (Peters et al. 2010; Monroe et al. 2016).

Wild-Type Factor IX

Normal human plasma factor IX exists in two allelic forms, with alanine or threonine in position 148 in the activation peptide. This dimorphism, however, is without any known functional implication. Some recombinant factor IX products provide the Ala-148 variant, while others have Thr in position 148 (see Table 18.3). The first recombinant factor

Table 18.3 Recombinant factor IX products

Product type	INN name	(Investigational) product name(s)	First approval (USA/EU)	Half-life (mean, h)	Production technology	Reference
Wild-type FIX	nonacog alfa	BeneFIX	1997	17–36	CHO cells, co-expression with furin, Ala-148 polymorphic variant	Harrison et al. (1998)
	nonacog gamma	Rixubis (<i>Bax326</i>)	2013	27	CHO cells, co-expression with furin, Ala-148 polymorphic variant	Dietrich et al. (2013)
	trenonacog alfa	IXinity (<i>IB1001</i>)	2015	24	CHO cells, Thr-148 polymorphic variant	Monroe et al. (2016)
Engineered FIX	eftrenonacog alfa	Alprolix (<i>FIX-Fc</i>)	2014	82	HEK cells, Thr-148 variant, Fc fused to FIX C-terminus, co-expression with Fc alone and with processing enzyme PC5	Peters et al. (2010)
	albutrepenonacog alfa	Idelvion (<i>F9-fusion protein</i>)	2016	92	CHO cells, Thr-148 variant, albumin fused to FIX C-terminus by cleavable linker	Metzner et al. (2009)
	nonacog beta pegol	Rebinyne, Refixia (<i>N9-GP</i>)	2017	96	CHO cells, Ala-148 variant, glycoPEGylation in activation peptide region	Østergaard et al. (2011)

IX was BeneFIX, developed at Genetics Institute. More than 15 years later, two other products have been licensed, Rixubis and IXinity, developed at Baxter and Inspiration Biopharmaceuticals, respectively. All three products are produced in CHO cells under serum-free conditions and undergo extensive downstream processing to remove impurities, including at least one virus eliminating step. For IXinity, the purification process includes one polishing step to specifically remove CHO-derived proteins (Monroe et al. 2016). Despite the differences in production and purification methods, the final products of these wild-type recombinant factor IX products are similar in terms of efficacy and pharmacokinetics.

Extended Half-Life Factor IX

Like factor VIII, also factor IX replacement therapy is limited by its relatively short half-life of about 24 h (see Table 18.3). For factor IX, engineering has proven more rewarding than for factor VIII. Currently, three products have been licensed that offer 3–four fold extended half-life (see Table 18.3 and references therein).

Alprolix has been developed by Biogen Idec and is a fusion between factor IX and the Fc fragment of IgG. The targeting of factor IX to the FcRn receptor prolongs the half-life to approximately 82 h. The fusion of the Fc fragment to the factor IX C-terminus yields a derivative in which the activated form still carries the Fc-moiety. The downside thereof is that the activity of this fusion protein is considerably lower than that of ‘wild-type’ Factor IX.

Idelvion has been developed by CSL Behring and is a fusion between factor IX and human albumin (Fig. 18.3c). The presence of the relatively large albumin at the C-terminus of the catalytic domain is incompatible with biological activity. This has been partially resolved by using a linker sequence that comprises the sequence of one of the two natural cleavage sites in factor IX (Metzner et al. 2009). Thus, proteolytic activation of this fusion protein involves three cleavages, the extra one serving to release the albumin moi-

ety. Nevertheless, this fusion protein displays reduced in vitro activity, possibly because albumin release is relatively slow or incomplete. Its reported half-life is ~92 h (Iorio 2017).

Rebinyne/Refixia has been developed by Novo Nordisk. In this product, recombinant factor IX is modified with a branched 40 kDa PEG. This modification is targeted to the native N-glycans in the activation peptide by enzymatic glycoconjugation. Because the PEG moiety is released with the activation peptide upon activation, the active form is the natural, unmodified factor IXa. The in vitro activity of this factor IX derivative seems normal, although this remains dependent of assay reagents used.

Several other engineered factor IX products are under development, including dalcinonacog alfa (ISU304, Catalyst Biosciences). This factor IX variant carries three point substitutions in the catalytic domain in order to enhance biological activity. Dalcinonacog alfa is currently being evaluated for subcutaneous use (Mahlangu et al. 2021).

Pharmacology

Like factor VIII, the term factor IX has been used before the protein had been identified. Factor IX was defined by its function to correct the clotting defect of plasma of patients suffering from hemophilia B. Biological activity has remained the basis for the quantification of factor IX, and concentrations are expressed in units, where 1 unit represents the amount of factor IX activity in 1 mL of normal human plasma. Plasma factor IX levels can also be expressed as % of normal. Thus, assessment of product potency, dosing, and pharmacokinetics are based on bioassays, and not on factor IX protein concentrations. Dosing is generally based on the empirical finding that 1 unit factor IX per kg body weight raises the plasma factor IX activity by 1% (or 0.01 unit/mL), according to the formula (Björkman and Berntorp 2001):

$$\text{dose (units / kg)} = 100 \times$$

$$(\text{required rise in units / mL factor IX})$$

Pharmacokinetics

The pharmacokinetics of factor IX have been reviewed in detail elsewhere (Björkman and Berntorp 2001; Iorio 2017). Systemic clearance is around 5 mL/h/kg for plasma-derived factor IX, and somewhat higher for recombinant wild-type factor IX. The distribution volume considerably exceeds the plasma volume, presumably because factor IX rapidly binds to the vascular surface. Elimination generally follows a bi-exponential pattern, with a terminal half-life of 20–34 h. Assessment of pharmacokinetics is mandatory for all individual factor IX products and seems more consistent than that of factor VIII (see Iorio 2017 and Table 18.3). Similar to factor VIII, factor IX pharmacokinetic data remain dependent on technical issues such as sampling time points, and the bioassays for the determination of the dose and post-infusion plasma levels. Despite this variability, it is evident that the engineered factor IX products do display a substantial half-life extension.

Pharmacodynamics

Hemophilia B is a congenital deficiency or dysfunction of factor IX which, dependent on the residual level of factor IX activity, is categorized as mild, moderate, or severe (Björkman and Berntorp 2001). Like factor VIII, normalization of factor IX levels in the circulation requires intravenous administration. The therapeutic range is narrow and similar to that of factor VIII.

For all licensed factor IX products, efficacy has been established for both on-demand and prophylactic treatment. On-demand treatment generally involves the administration of 20–100 units/kg, depending on the bleeding type. Long-term prophylaxis involves regular treatment at a dose of 20–40 units/kg, with intervals of 3–4 days. For extended half-life products, various regimens have been assessed, varying from 10 units/kg every 7 days for one product to 75 units/kg every 14 days or 100 units/kg every 10 days for other products (Young and Mahlangu 2016). Thus, no general recommendation can be given so far. For the time being, dosing should strictly adhere to the product-specific recommendations for the individual products given in the Summary of Product Characteristics as issued by regulatory authorities such as EMA.

Unlike factor VIII, an immune response against factor IX is rare. Hypersensitivity and allergic reactions have been reported, and factor IX inhibitory antibodies may occur in a very small proportion of patients (1% or less). Antibodies against hamster proteins have been reported for one particu-

lar product (IXinity), but this has been resolved by introducing an additional step to further reduce these impurities (Monroe et al. 2016).

Pharmaceutical Considerations

The factor IX concentration in human plasma is approximately 4 µg/mL. However, like factor VIII, activity units are used for factor IX. The potency of factor IX products is expressed in International Units (IU) relative to an international standard established by the World Health Organization. Most products are available in fillings ranging between 250 and 3000 IU in order to provide appropriate, body-weight guided, dosing for children and adults. Current products are freeze dried and need to be reconstituted before infusion. Most products are stabilized in a sucrose-containing formulation and have a shelf life of 24–36 months upon storage at 2–8 °C. For most products, data are available that establish stability at room temperature for 6–24 months.

As for impurities, most products may contain low amounts of residual proteins from the host expression system. With the exception of Alprolix, which is expressed in a human cell line, these products may cause side effects in patients which hypersensitivity against rodent proteins. To enhance safety further, the downstream processing of most recombinant factor IX products includes one or more virus-eliminating steps, usually including nanofiltration.

Clinical Considerations

The sole indication for recombinant factor IX is congenital hemophilia B. For all factor IX products, appropriate treatment protocols have been established as part of the licensing procedure. Like for hemophilia A, there is an increasing interest in personalized prophylactic treatment based on the pharmacokinetic profile of individual patient/product combinations (Iorio 2017). This remains challenging for the extended half-life products, because of the lack of uniform, robust assays for monitoring these engineered factor IX variants (Kitchen et al. 2017).

An additional complication is that the Fc- and albumin fusion proteins display reduced activity, indicating that patients are treated with factor IX that is partially inactive. This might seem irrelevant because dosing is based on activity (Peters et al. 2010). On the other hand, this introduces an extra, assay-dependent variable in personalized, pharmacokinetics-based dosing. One important implication is that patients cannot be switched from one extended half-life product to another while maintaining the same dosing schedule.

Other Hemostatic Proteins

While a variety of recombinant factor VIII and IX products is available from multiple biotechnology companies, other hemostatic proteins have received limited attention. For some proteins, no more than one or two products have been licensed so far. These are reviewed in the present section.

Recombinant Factor VIIa

Factor VIIa is the enzyme that triggers the initiation phase of thrombin generation and acts directly upstream of factor X in the cascade. Based on theoretical considerations, it has been proposed that the presence of high concentrations of factor VIIa should have the potential of activating factor X to a sufficient extent to bypass the need for factor VIII or factor IX in the coagulation cascade (see Fig. 18.1). Once this concept had been verified (Hedner and Kisiel 1983), the use of factor VIIa became an option for treatment of bleeding in patients with inhibitory antibodies against factor VIII or IX, and a few other bleeding disorders (see Clinical considerations).

The factor VII concentration in human plasma is low (0.5 µg/mL) and as such is insufficient to provide a source for large amounts of factor VIIa. This made factor VIIa an attractive target for production by recombinant technology. Factor VII is a glycoprotein of approximately 57 kDa, which has the same domain structure as factor IX (Fig. 18.3). It shares the same requirement for γ -carboxylation in its Gla-domain at the N-terminus of the mature protein. Recombinant factor VII has been developed at Novo Nordisk and is produced in BHK cells under serum-free conditions. During downstream processing, single-chain factor VII is converted into two-chain factor VIIa by autoactivation (Hedner 2006). Recombinant factor VIIa (INN name eptacog alfa) has been licensed since 1996 under the product name NovoSeven.

A nearly identical product (eptacog beta, investigational name LR769) has been developed at LFB Biotechnologies and is licensed in the USA and EU since 2020 and 2022 under the names Sevenfact and Cevenfacta, respectively. It differs from most other recombinant products in that it is harvested from the milk of transgenic rabbits expressing human factor VII. Downstream processing results in fully active factor VIIa which carries all relevant post-translational modifications (Chevreux et al. 2017). Another bio-similar is produced by AryoGen Biopharma. This product (AryoSeven) has only been licensed in Iran so far. Marzeptacog alfa is an engineered factor VIIa, with four amino acid substitutions to enhance stability and activity, and is under investigation for subcutaneous administration (Mahlangu et al. 2021). Another engineered variant (vatrep-

tacog alfa) has been designed to enhance biological activity, but this variant proved to be immunogenic due to the amino acid substitutions made, and phase III trials have been terminated (Mahlangu et al. 2015). Although the recent advent of transgenic factor VIIa holds promise for the future, NovoSeven is the sole recombinant factor VIIa product that is widely used so far.

Pharmacology

Factor VIIa normally activates factor X when bound to tissue factor at the site of vascular injury. NovoSeven dosing, however, is in large excess over physiological concentrations. Under such conditions, factor VIIa is believed to activate factor X on the surface of activated platelets in a tissue factor-independent manner (Hedner 2006). Factor VIIa potency and dosing is based on bioassays but, unlike factor VIII and IX, is expressed in terms of protein concentration, and not in units. Like other coagulation factors, Factor VIIa therapy requires intravenous administration. The pharmacokinetics of recombinant factor VIIa has been assessed in various patient groups. In non-bleeding patients, the overall mean clearance is approximately 30 mL/h/kg, and the half-life is around 2–3 h (Björkman and Berntorp 2001). For hemophilia A or B patients with inhibitory antibodies, the recommended dosage is 90 µg/kg every 2 h until hemostasis is achieved. Dosage is slightly different for other indications (see section “Clinical Considerations”).

Pharmaceutical Considerations

NovoSeven is available in fillings of 1–8 mg per vial. The product is freeze-dried and needs to be reconstituted in a specific histidine-containing diluent before use. The product is stable for 3 years at room temperature. As for impurities, downstream processing includes a virus inactivating solvent/detergent step.

Clinical Considerations

Apart from treatment of hemophilia A and B with inhibitory antibodies, recombinant factor VIIa is also indicated for treatment of acquired hemophilia due to antibodies in non-hemophilic patients. Another indication is congenital factor VII deficiency. The recommended dosage then is 15–30 µg/kg every 4–6 h. NovoSeven is also indicated for the platelet-based bleeding disorder Glanzmann Thrombasthenia. The recommended dosage then is 90 µg/kg every 2–6 h, until hemostasis has been achieved (Hedner and Ezban 2008). The most common and serious adverse reactions are thrombotic events. As for Sevenfact/Cevenfacta, the only indication is hemophilia with inhibitory antibodies. Due to its transgenic origin, this product is not recommended for patients with known hypersensitivity to rabbits.

Recombinant von Willebrand Factor

Von Willebrand factor is one of the largest proteins in circulation. Monomeric von Willebrand factor is synthesized as a preproprotein, with a signal peptide of 22 amino acids, a propeptide that is unusually large (741 amino acids) and a mature subunit of 2015 amino acids (for review, see Castaman and Linari 2017). Monomers form tail-to-tail dimers via a cysteine-rich region at the C-terminus. These dimers can multimerize by the formation of cysteine bridges at the N-terminus of the mature subunit in a process that requires the presence of the propeptide. The molecular size of the von Willebrand factor thus can vary between a dimer of 500 kDa and multimers of up to 20,000 kDa. The primary function of the von Willebrand factor is to promote platelet aggregation and adhesion, which is essential for appropriate thrombus formation (Fig. 18.1b). The secondary function is to carry factor VIII in the circulation and thereby to prevent its premature clearance (Pipe et al. 2016; Castaman and Linari 2017).

It seems evident that the size of this protein and the complexity of its processing into dimers and multimers make the production of a recombinant von Willebrand factor a challenge. Nevertheless, this has already been achieved in the 1980s at Genetics Institute by the co-expression of von Willebrand factor and factor VIII. While the factor VIII products resulting from co-expression (Recombinat and Advate, see Table 18.2) were depleted of the von Willebrand factor, this byproduct has subsequently been developed into a separate product. Downstream processing includes *in vitro* processing with recombinant processing enzyme (furin) to remove the propeptide and expose optimal factor VIII binding (Turecek et al. 2009). This recombinant product (INN name vonicog alfa) has first been licensed as Vonvendi in the US in 2015 and is called Veyvondi in the EU. It is the first recombinant von Willebrand factor available as an alternative to the current plasma-derived counterparts (Franchini and Mannucci 2013).

Pharmacology and Pharmaceutical Considerations

Recombinant von Willebrand factor serves to control bleeding episodes in patients with severe type-III deficiency. The deficiency of von Willebrand factor in these patients implies not only a defective platelet aggregation and adhesion but also abnormally fast clearance of their endogenous factor VIII. Therefore, factor VIII needs to be supplemented too, usually to a level of 35–50% of normal, in order to achieve hemostasis (Mannucci 2004).

The concentration of recombinant von Willebrand factor is expressed in terms of biological activity by its effect on platelet aggregation in the presence of ristocetin (so-called

‘ristocetin cofactor activity’). Potency is expressed in International Units (IU) and is based on the International Standard for von Willebrand factor concentrate. Vonvendi is available as lyophilized powder for reconstitution in fillings of 650 and 1300 IU and needs to be administered intravenously. Its shelf-life is 36 months at 3–5 °C or 12 months at room temperature. Being derived from CHO cells under serum- and protein-free conditions, the most relevant impurities may represent residual hamster protein, which might cause hypersensitivity reactions in some recipients. The pharmacokinetics have been studied extensively (Gill et al. 2015), both for recombinant von Willebrand factor alone and in combination with recombinant factor VIII (see under Clinical considerations). The overall clearance was 3 mL/h/kg and half-life approximately 22 h. In the phase III study, the initial dosage was 40–80 IU/kg, followed by 40–60 kg every 8–24 h if clinically required.

Clinical Considerations

The vast experience with the treatment of severe von Willebrand’s disease is based on the use of plasma-derived products, in particular concentrates that contain both von Willebrand factor and factor VIII. The recommended regimen is 30–50 IU/kg von Willebrand factor while keeping the trough factor VIII level > 30–50% of normal (Mannucci 2004). In Vonvendi, the sole active component is the recombinant von Willebrand factor. To control bleeding, the first dosage should therefore be combined with recombinant factor VIII. Because the recombinant von Willebrand factor sustains the stability of the patient’s endogenous factor VIII, co-administration with factor VIII for subsequent infusions might prove unnecessary (Gill et al. 2015). For the moment, the burden of co-administration and dual monitoring seems to be prohibitive for general use in less specialized centers. Ongoing post-marketing trials serve to clarify further the exact place of recombinant von Willebrand factor in the management of this complex bleeding disorder (Franchini and Mannucci 2016).

Recombinant Factor XIII

Factor XIII is the pro-enzyme of a transglutaminase that, after activation by thrombin, reinforces fibrin polymers by the formation of γ -glutamyl- ϵ -lysyl amide cross-links. A congenital deficiency of factor XIII is associated with severe bleeding due to impaired thrombus stability (see Fig. 18.1b). Factor XIII is a hetero-tetramer comprising two catalytic A-subunits of 82 kDa and two carrier B-subunits of 73 kDa, which are linked by noncovalent interactions (Komaromi et al. 2011). The A- and B-subunits are encoded by different genes, and in plasma, the B-chain circulates in excess over

the A-chain. An intracellular form of factor XIII is present in platelets, macrophages, and other cells and is a homodimer of two A-subunits only. Most patients with factor XIII deficiency have a defect in the A-subunit, which makes this a suitable target for recombinant factor XIII substitution therapy (Inbal et al. 2012).

Pharmacology and Pharmaceutical Considerations

Recombinant factor XIII A-subunit has been developed at ZymoGenetics and Novo Nordisk and is expressed in *Saccharomyces cerevisiae* under protein-free conditions. The purified yeast-derived protein is a dimer of two non-glycosylated factor XIII A-subunits (Lovejoy et al. 2006), which upon infusion spontaneously associates with B-subunits to form the factor XIII heterotetramer in the circulation. The product (INN name catridecacog) has been licensed in 2012 and is available under the product name Tretten in the US and as NovoThirteen elsewhere.

The potency of recombinant factor XIII is expressed in International Units (IU) and is based on the International Standard for factor XIII concentrate. NovoThirteen is available as lyophilized powder for reconstitution in vials of 2500 IU, to be reconstituted in physiological saline for intravenous administration. The shelf-life is 24 months at 2–8 °C. Pharmacokinetic studies have shown that the half-life varies between 6 and 9 days (Lovejoy et al. 2006). Prophylactic treatment has proven efficacious and safe using a regimen of 35 IU/kg once monthly (Inbal et al. 2012).

Clinical Considerations

Recombinant factor XIII is the first recombinant product for a rare bleeding disorder. So far, plasma-derived, heterotetrameric factor XIII has been used for the treatment of congenital factor XIII deficiency. Due to the lack of post-translation glycosylation, the yeast protein is non-identical to the natural A-subunit homodimer. Nevertheless, this has not resulted in any detectable immunogenicity so far. For a majority of patients, therefore, recombinant A-subunit dimer proves a viable alternative for plasma-derived factor XIII.

Recombinant Antithrombin

Antithrombin belongs to the class of serine protease inhibitors ('serpins'), and as such might be considered as not being a coagulation factor in the strict sense. However, antithrombin is the major inhibitor of factor IXa, factor Xa, factor XIa, and thrombin and thereby plays a regulatory role at multiple levels in the coagulation cascade. Antithrombin is a single-chain glycoprotein and has a molecular mass of 58 kDa. Its inhibitory potential is greatly enhanced by binding to hepa-

rin or other glycosaminoglycans. This drives antithrombin, like other serpins, into a conformation that favors the interaction with its target proteases (Huntington 2003). A partial deficiency of antithrombin reduces the downregulation of the coagulation cascade and thereby enhances the risk of thrombosis. In antithrombin deficiency, prophylaxis usually consists of standard antithrombotic therapy using heparin or small molecules that inhibit coagulation (Mackman 2008). However, the inherent bleeding risk thereof makes such therapy undesirable for use in high-risk situations such as surgery or delivery. Suppletion of antithrombin then provides an alternative treatment.

Recombinant antithrombin is licensed in the EU and US since 2006 and 2009, respectively. The product has been developed by Genzyme Transgenics (GTC Biotherapeutics) and is available as ATryn (INN name antithrombin alfa). ATryn is particularly remarkable from a biotechnological point of view, because it is harvested from the milk of transgenic goats (Edmunds et al. 1998). This technology, which is introduced in Chap. 9, has proven feasible for the expression of a wide range of therapeutic proteins in milk or other body fluids (Lubon et al. 1996, see also Table 9.4). During further development, however, transgenic technology had to deal with numerous issues, including stability of transgenic inheritance, appropriate post-translational modification and stability of heterologous proteins in milk, and regulatory affairs (Lubon et al. 1996). Upon its approval by EMA and FDA, ATryn became the first biopharmaceutical that took all hurdles of production in transgenic animals. Since then, a few more transgenic biopharmaceuticals have been licensed, including recombinant factor VIIa (see above).

Pharmacology and Pharmaceutical Considerations

The potency of ATryn is expressed in International Units, which relate to the International Standard for antithrombin in concentrate as established by WHO. The unitage is based on in vitro inhibitory activity in the presence of heparin. ATryn is available as lyophilized powder for reconstitution and infusion, in fillings of 1750 IU per vial. When stored at 2–8 °C, the shelf-life is 4 years. The transgenic antithrombin in ATryn is identical to plasma-derived antithrombin, with the exception of its glycosylation, which is less complex and comprises fewer sialic acid moieties (Edmunds et al. 1998). Rigorous pharmacokinetic studies have not been reported, but the estimated mean elimination half-life is 10 h, which is six-fold shorter than that of the fully glycosylated antithrombin from human plasma. Therefore, dosing recommendations for plasma-derived and transgenic antithrombin are different. Dosing aims to maintain plasma levels between 80 and 120% of normal. For ATryn, the recommended dosing is (Tiede et al. 2008):

$$\text{Loading dose (IU / kg)} = \left(\frac{100 - \text{pretreatment antithrombin level in\%}}{\text{antithrombin level in\%}} \right) / 2.28$$

$$\text{Maintenance (IU / kg / hr)} = \left(\frac{100 - \text{pretreatment antithrombin level in\%}}{\text{antithrombin level in\%}} \right) / 10.22$$

The loading dose can be administered as a bolus, while maintenance requires continuous infusion.

Clinical Considerations

ATryn is indicated for the prophylaxis of venous thrombosis in surgery in adult patients with congenital antithrombin deficiency. In the absence of sufficient data, ATryn initially has not been recommended for use during pregnancy. Subsequent studies have generated additional support for using ATryn in both perioperative and peripartum settings (Paidas et al. 2014). One concern could be the potential immunogenicity of the transgenic protein due to the glycosylation differences between transgenic and native human antithrombin. However, no antibodies have been reported, neither to the transgenic protein nor to any other goat protein (Paidas et al. 2014).

Recombinant Thrombolytic Agents

The need for thrombolytic agents dates from the 1970s, when myocardial infarction was recognized as being caused by the rupture of an atherosclerotic plaque in a coronary artery, followed by the formation of an occluding thrombus (Lusis 2000). Recanalization of the occluded vessel requires thrombus dissolution, which can be achieved by activating the fibrinolytic system (see Fig. 18.1c). The first thrombolytic agent used was streptokinase, a bacterial plasminogen activator isolated from *Streptococcus haemolyticus*. This agent has been widely used in the treatment of myocardial infarction and as such has developed into a worldwide blockbuster. However, the use of a bacterial protein implies the risk of immunogenicity, and this may hamper repeated dosing. The search for a human plasminogen activator has resulted in the identification of a tissue-type plasminogen activator (t-PA). Its low concentration in tissues and in the blood is prohibitive for obtaining natural t-PA in substantial amounts. This made t-PA an obvious candidate for production by recombinant technology (for review, see Collen and Lijnen 2005).

Recombinant Tissue-Type Plasminogen Activator (t-PA)

The primary structure of human t-PA has been established since the cloning and expression of its cDNA (Pennica et al. 1983). The mature protein comprises an N-terminal region that is called the finger domain, followed by a single EGF-like domain, two so-called kringle domains, and the catalytic domain (see Fig. 18.4a). There are four sites for N-linked glycosylation, one in kringle-1, two in kringle-2, and one in the protease domain. Human t-PA is a single-chain protein with a molecular mass of about 70 kDa. It is converted into a two-chain form by plasmin, by cleavage at the junction between the kringle-2 and the catalytic domain. While the two-chain form represents the fully activated enzyme, the single-chain form displays very similar fibrinolytic activity. Both forms of t-PA share high-affinity interaction with fibrin and inhibition by plasminogen activator inhibitor-1 (PAI-1) (Rijken et al. 1982).

Wild-Type Recombinant t-PA (Alteplase)

Wild-type t-PA has initially been expressed in *Escherichia coli* (Pennica et al. 1983). However, the numerous (17) internal disulfide bridges and multiple glycosylation sites make production in mammalian cells more efficient. Recombinant t-PA (INN name alteplase) has been developed at Genentech and is produced in CHO cells. Alteplase displays the fibrin-specificity of natural t-PA, which is mediated by the finger and kringle-2 domain. The biological half-life is only 5–6 min. Half-life is limited by the rapid inhibition by PAI-1 and by the interaction of the finger and/or EGF-like domains with clearance receptors (Collen and Lijnen 2005). Alteplase has first been licensed in 1987 and is available under multiple product names, including Activase (Genentech) and Actilyse (Boehringer Ingelheim).

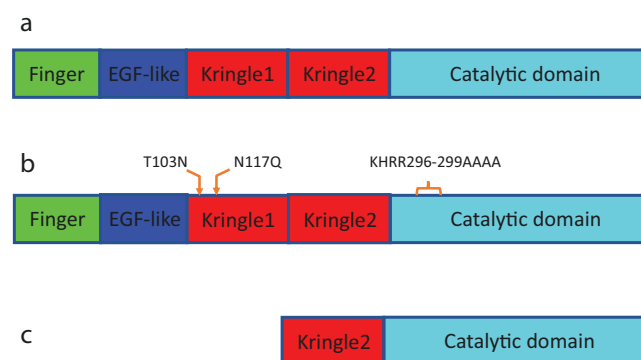


Fig. 18.4 Structure of recombinant tissue-type plasminogen activator. Panel (a) represents the domain structure of full-length t-PA (alteplase). Panel (b) displays the variant tenecteplase, and panel (c) the truncated variant reteplase. Further details are given in the text. EGF: epidermal growth factor

Engineered Recombinant t-PA

Tenecteplase is the INN name of a t-PA variant that has been engineered at Genentech, with the aim to prolong half-life, while maintaining the fibrin specificity of natural t-PA. This has been accomplished by a few substitutions in the full-length protein (see Fig. 18.4b). The Thr103 to Asn (T103N) substitution creates a new N-linked glycosylation site, and the Asn117 to Gln (N117Q) mutation eliminates a high-mannose glycosylation site in the kringle-1 domain. In addition, the amino acids Lys295-His-Arg-Arg299 have been replaced by four Ala residues. The modified glycosylation in the kringle-1 domain serves to reduce clearance, while the KHRR296-299AAA substitutions confer increased resistance against inhibition by PAI-1 (Keyt et al. 1994). Tenecteplase is produced in CHO cells and has first been licensed in the US in 2000. It is available under the product names TNKase and Metalyse.

Retepase is the INN name for a truncated variant of t-PA which consists of only the kringle-2 and the catalytic domain (see Fig. 18.4c). This variant was developed at Boehringer Mannheim, with the objective of eliminating the domains that drive clearance, while conserving the kringle-2 domain for fibrin-specificity. This major truncation allows for production in *Escherichia coli*. Retepase is a single-chain, non-glycosylated protein with a molecular mass of 39 kDa. Its downstream processing includes extraction from inclusion bodies and in vitro renaturation and refolding (Moser et al. 1998; Simpson et al. 2006). Retepase was first licensed in 1996 and is available under the product names Rapsilysin and Retevase.

A variety of other t-PA variants has been designed, including lanoteplase. Lanoteplase has been developed at Genetics Institute. It lacks the finger and EFG-like domains, as well as the glycosylation in position Asn117 (Collen and Lijnen 2005). Like other, more recently engineered t-PA variants, lanoteplase has not been licensed and therefore remains beyond the scope of this chapter.

Pharmacology

Multiple methods are being used for the quantification of t-PA. First, potency is expressed in terms of International Units (IU), which reflects biological activity against the International Standard as established by WHO. Alternatively, t-PA concentration can be defined by protein content and expressed in mg. Which method is used for potency labeling varies between products. For reteplase, potency is expressed in units (U) using a product-specific reference. For patient monitoring or for pharmacokinetic studies, t-PA concentrations are usually based on immunological assays.

Pharmacokinetics

Assessment of the pharmacokinetics of t-PA is complex. It is reflecting a combination of 'regular' plasma elimination of free t-PA, the adsorption of t-PA to insoluble fibrin, the neutralization by PAI-1, and the elimination of t-PA/PAI-1 complexes. The reader is referred to the Summary of Product Characteristics of the individual products for details (EMA). As a general parameter, most studies just use the biological half-life as estimated from levels of circulating t-PA. In these terms, the half-life of alteplase is 5–6 min, whereas the half-life of tenecteplase and reteplase is 17–20 min and 14–18 min, respectively (Collen and Lijnen 2005).

Pharmacodynamics

The recombinant plasminogen activators differ from streptokinase in that they are fibrin-specific. Fibrin has a high affinity for both t-PA and plasminogen and serves as a surface for local plasminogen activation. In contrast to free plasmin, fibrin-bound plasmin is relatively insensitive to inactivation by α 2-plasmin inhibitors. These interactions make fibrin the central regulator of its own degradation (Collen and Lijnen 2005). Normally, this subtle mechanism is triggered adequately by the small amounts of t-PA in plasma (5–10 ng/mL).

Numerous clinical studies have addressed recombinant t-PA in acute myocardial infarction, usually in comparison with a standard streptokinase regimen. Due to the difference in pharmacokinetics, dosage regimens are product-specific. For alteplase, a typical regimen is a total of 100 mg, of which 60 mg during the first hour, and 20 mg over the second hour, and 20 mg over the third hour. An alternative is 100 mg divided over a bolus of 15 mg, followed by 0.75 mg/kg over 30 min, and 0.5 mg/kg over 60 min (Collen and Lijnen 2004).

In contrast, tenecteplase usually is administered as a single intravenous bolus of 30–50 mg (6000–10,000 IU), dependent on body weight (Guillermin et al. 2016). For reteplase, a recommended regimen is a double bolus (10 U + 10 U, 30 min apart), independent of body weight (Simpson et al. 2006). Apparently, the engineering to extend half-life of tenecteplase and reteplase indeed resulted in the intended dosing advantage.

In thrombolytic therapy, t-PA levels are achieved that are in large excess (up to 500-fold) over the physiological concentration. This carries the inherent risk that t-PA also acts in a fibrin-independent manner, and degrades fibrinogen (fibrinogenolysis). This may lead to systemic fibrinogen shortage and concomitant bleeding risk. Bleeding is the most common complication of thrombolysis, also because it is usually combined with anticoagulant therapy to prevent re-occlusion. This may vary between superficial bleeding at the site of injection and more severe episodes, including intracranial bleeding.

Pharmaceutical Considerations

Alteplase, tenecteplase and reteplase are lyophilized products that need to be reconstituted for intravenous infusion. Shelf-life is at least 2 years at temperatures not exceeding 25 °C. Once reconstituted, alteplase can be further diluted with a physiological saline solution to facilitate continuous infusion. Usually, thrombolytic therapy is combined with continuous infusion of heparin. This should not be co-administered through the same cannula, because of solubility issues, in particular for reteplase.

As for impurities, alteplase and tenecteplase are produced in CHO cells and may include residual hamster protein. Reteplase is produced in *Escherichia coli* and is subjected to a validated process of denaturation and refolding to produce the active fibrinolytic enzyme. Reteplase has a lower affinity for fibrin than alteplase and tenecteplase. It also displays lower in vitro fibrinolytic activity than alteplase (Simpson et al. 2006). Because of its different unitage used for potency labeling, the content of active agent in reteplase cannot be directly related to that of alteplase or tenecteplase.

Clinical Considerations

The primary indication for alteplase, tenecteplase, and reteplase is acute myocardial infarction. Alteplase has further been licensed for use in acute pulmonary embolism and acute ischaemic stroke. For tenecteplase and reteplase, studies are addressing the extension into these indications too. For the treatment of stroke, it is essential to start therapy only after the prior exclusion of intracranial bleeding by appropriate imaging techniques.

In the majority of clinical trials, the recombinant agents have been compared with standard streptokinase therapy, often in combination with heparin or other anticoagulants, to prevent re-occlusion (Collins et al. 1997). Overall, all agents seem to offer adequate thrombolytic therapy of acute myocardial infarction, with comparable survival rates and bleeding complications. In the absence of a direct comparison between recombinant thrombolytic agents, it remains difficult to conclude that one agent would be preferable over another. A meta-analysis suggests that tenecteplase and alteplase are equally effective in acute coronary syndrome, with a slightly lower bleeding risk for tenecteplase (Guillermin et al. 2016). It should be noted, however, that clinical studies are complex and require the inclusion of large numbers of patients to reach satisfying conclusions for all individual indications. Therefore, the current thrombolytic agents continue to be the subject of clinical studies to further explore their therapeutic potential in life-threatening thrombotic complications.

Concluding Remarks

In the early 1980s, the advent of biotechnology provided the perspective of unlimited access to recombinant coagulation factors and thrombolytic agents. This was particularly promising for low-abundance proteins such as t-PA, and factors VII and VIII, for which the medical need was by far exceeding the availability of pharmaceutical production from human sources. Now, four decades later, one may wonder whether biotechnology actually has fulfilled its initial promise in this field. It remains difficult to give a fully unequivocal answer at this point.

On the positive side, there are some major achievements. The most prominent example is t-PA. Its cDNA was cloned in 1983, and recombinant t-PA became available as a life-saving product already 4 years later. It is further remarkable that for recombinant factor VIII, being a much more complex protein than t-PA, the gap between cloning and licensing could be closed in only 8 years. Another success is activated factor VII. Recombinant technology has been instrumental in obtaining this trace protein in sufficient amounts to meet the clinical need. More recently, we have seen examples of how protein engineering may generate biopharmaceuticals that display more favorable pharmacokinetics than their natural counterparts.

At the same time, however, some limitations remain apparent. For instance, recombinant t-PA now is the generally established agent for thrombolytic therapy in the US and EU, but not in territories where t-PA is not affordable, and streptokinase is still being used. Another example is factor VIII. Since the introduction of recombinant factor VIII supplies has been a limiting factor, and prices have remained prohibitive for many countries. While factor VIII is included in WHO's List of Essential Medicines, the majority (~75%) of the world's patients still lack appropriate access to hemophilia care, despite intense efforts to maximize the production of conventional factor VIII from human plasma. Apparently, recombinant factor VIII did not meet the full medical need. In this regard, one may question why so many new recombinant factor VIII products have been recently developed without taking the affordability issue into account. Moreover, with the exception of factor XIII deficiency, the rare bleeding disorders have remained dependent on blood-derived products, with their inherent limited supply. The initial expectation was that plasma-derived coagulation factors may soon become obsolete. It is evident that this optimism has been premature.

The next few decades offer excellent perspectives on fulfilling the initial promise of biotechnology. It may become possible to replace costly mammalian cell expression with cheaper technology. For relatively simple proteins such as t-PA, expression in transgenic plants has proven feasible.

Production of transgenic animals has, after many hurdles, resulted in the first tangible products on the market. Moreover, for most of the hemostatic proteins, the dominant patents have expired, thus opening the way towards more affordable biosimilars (cf. Chap. 11). Finally, protein engineering continues to generate therapeutics that display improved biological activity at a much lower dosage. It remains an attractive challenge to a new generation of researchers to accomplish these goals in the near future.

Self-Assessment Questions

Questions

1. *Proteolytic Processing*

Furin-like enzymes play a role in the post-translational modification of a several hemostatic proteins.

- Give three examples and describe the relevance of processing in these proteins.
- How is this modification accomplished in biotechnological production?

2. *Transgenic animal bioreactors*

A variety of transgenic animals has been used to express hemostatic proteins in their milk, including rabbits, goats, sheep, pigs and cows. Recombinant antithrombin (ATryn) is produced in goats.

- ATryn differs from plasma-derived antithrombin. What is the major difference? How could this be caused?
- Would you consider using goats as bioreactor for producing transgenic factor VIII or IX, or would you prefer an alternative? Why?

3. *Extended half-life factor IX*

The factor IX-albumin fusion protein is one of the available products with extended half-life.

- What is the mechanism underlying the half-life prolongation?
- Are there particular disadvantages or limitations of this particular fusion strategy?
- What are the implications for patients switching from normal factor IX to this fusion product?

4. *Thrombolytic agents*

- Describe the mechanism by which fibrinolysis is under local control.
- What is the main adverse effect of thrombolysis, and how is this caused.
- Retepase differs from other thrombolytic agents in that it is produced in *E. coli*. What are the potential advantages or disadvantages of this approach?

Answers

1. *Proteolytic processing*

- Three examples are: (1) factor IX, wherein the propeptide that is needed for appropriate γ -carboxylation, needs to be cleaved off in order to generate the Gla-

domain as the natural N-terminus of the mature factor IX, (2) von Willebrand factor, wherein the propeptide is important for the formation of multimers, but is removed prior to secretion in order to facilitate its function as carrier of factor VIII in the circulation, and (3) factor VIII, which is processed into a two chain heterodimer as in plasma-derived, natural factor VIII; the role of this cleavage is unclear, and one recombinant product is deliberately constructed to be single-chain (see Table 18.2).

- If cleavage by endogenous processing proteases is limiting (as for instance in CHO cells), the usual approach is to co-express furin (or a related protease) in the same cells, or to perform in vitro maturation using a recombinant processing protease (as in recombinant von Willebrand factor).

2. *Transgenic animal bioreactors*

- The major difference between ATryn and plasma-derived antithrombin is the glycosylation, with less complicated glycans that carry fewer end-standing sialic acid moieties. This suggests that glycosylation in goat mammary gland cells is incomplete. Apparently, this translates into a shorter half-life.
- Half-life of factor VIII or IX is a key issue because these proteins are predominantly used for long-term prophylaxis. The use of transgenic goats would imply a risk of undesirably short half-life. Factor VIII and IX have been expressed in pigs, and glycosylation patterns seem less aberrant than in goats. Establishing correct glycosylation is a crucial element in assessing the potential benefit of transgenic technology for proteins that are to be used in non-acute situations.

3. *Extended half-life factor IX*

- The factor IX-albumin fusion binds to the FcRn receptor via its albumin moiety. Due to the binding to this recycling receptor, the fusion protein escapes endosomal degradation after endocytosis and recycles back to the circulation.
- The disadvantage is that factor IX is inactive as long as albumin remains fused to its catalytic domain. This has been solved by creating a cleavable linker. However, partial cleavage of the linker at the site of injury will reduce biological activity.
- For the patient, dosing intervals will be significantly longer. Alternatively, trough levels of factor IX will be higher, which adds protection against spontaneous bleeding. However, because monitoring is more complex than for other factor IX products, it is more difficult to develop an individualized dosing regimen.

4. *Thrombolytic agents*

- Fibrinolysis is controlled by fibrin, which binds t-PA and plasminogen (see text), and thereby limits plasmin formation to the site of the occluding thrombus.

- (b) The main adverse effect of thrombolysis is bleeding. This is largely due to insufficient fibrin-specificity, leading to the degradation and consumption of fibrinogen (fibrinogenolysis).
- (c) The advantage of production in *E. coli* is the lower production cost. The disadvantage is the need for denaturation and refolding. Reteplase lacks a few domains of normal t-PA, including the fibrin-binding finger domain. Therefore, its affinity for fibrin is lower, which theoretically reduces its fibrin-specificity.

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