Recombinant Activated Factor VII (rFVIIa): Characterization, Manufacturing, and Clinical Development

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ABSTRACT

Recombinant activated coagulation factor VII (rFVIIa) (NovoSeven®) was developed for treatment of bleeding in hemophilia patients with inhibitors (antibodies) against factors VIII or IX. rFVIIa initiates the coagulation cascade by binding to tissue factor at the site of injury and causes the formation of sufficient amounts of thrombin to trigger coagulation. Patients with a variety of other coagulation deficiencies than hemophilia characterized by an impaired thrombin generation and life-threatening bleeding have been reported as successfully treated with rFVIIa. Data are now entered into clinical registries established to further monitor this experimental treatment with NovoSeven®. rFVIIa is produced free of any added human protein. The amino acid sequence of rFVIIa is identical to plasma-derived FVIIa (pdFVIIa). Posttranslational modifications (i.e., γ-carboxylations, N- and O-glycosylations) are qualitatively identical in pdFVIIa and rFVIIa although some quantitative differences exist. The activities of rFVIIa and pdFVIIa are indistinguishable. Manufacturing of rFVIIa involves expression in baby hamster kidney (BHK) cells followed by purification, including three ion-exchange and one immunoaffinity chromatography steps. The last anion-exchange chromatography step ensures completion of the autoactivation of recombinant factor VII (rFVII) to rFVIIa. This review describes the mechanism of action, characterization, manufacturing, and preclinical and current clinical evidence for the efficacy and safety of rFVIIa.

KEYWORDS: Factor VIIa, rFVIIa, recombinant expression, in vitro activation, biological activity

Objectives: Upon completion of this article, the reader should be able to (1) realize the structural and enzymatic similarities of plasma-derived factor VIIa (pdFVIIa) and recombinant factor VIIa (rFVIIa) and (2) describe the mechanism of action of rFVIIa.
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Recombinant activated coagulation factor VII (rFVIIa) (eptacog alpha) is the generic name for NovoSeven®. rFVIIa is now registered in more than 50 countries worldwide for the treatment of spontaneous and surgical bleeding episodes in patients with inhibitors (antibodies) against factor VIII (FVIII) or factor IX (FIX).[1–7]

During the last decades, the treatment of congenital and acquired hemophilia patients has progressed from the use of cryoprecipitate to purified plasma-derived (pd) factor concentrates. Furthermore, the development of highly purified recombinant coagulation factors, free of human viruses, has provided a major step in improvement of safety and convenience for these patients.[8–10]

Effective treatment of life- and limb-threatening bleeding episodes in inhibitor patients is still a major challenge. Surgery presents a particular challenge to hemostasis management, and, until recently, inhibitor patients were often denied elective surgical procedures.

Clinical trials have demonstrated that administration of rFVIIa in doses of 90 μg/kg every 2 hours effectively (80 to 90%) and safely ensures hemostasis in hemophilia patients with inhibitors.[1,2,5–7,11] In addition, FVII-deficient patients have been successfully treated with rFVIIa in doses of 20 to 30 (μg/kg every 6 hours.[12–14] Inhibitor- and FVII-deficient patients treated with rFVIIa can undergo major surgery with minimal risk of uncontrolled hemorrhage and complications.[2,13,15,16]

Exogenous rFVIIa forms complexes with tissue factor (TF) exposed at the site of injury. The subsequent limited amount of thrombin generation enhances both platelet activation and further thrombin generation on the surface of the activated platelets at the site of injury. This localized effect may ensure safety of this drug with no global activation of the coagulation system. Patients with a variety of coagulation deficiencies characterized by an impaired thrombin generation and uncontrollable life-threatening bleedings are reported successfully treated with rFVIIa.[17] However, the efficacy and safety of treatment with rFVIIa outside the current indication should be confirmed in randomized placebo-controlled clinical trials.

This article describes the mechanism of action, characterization, and manufacturing of rFVIIa. Also briefly mentioned are the preclinical characteristics, the pharmacokinetics, and the current clinical evidence for the efficacy and safety of treatment with rFVIIa.

MECHANISM OF ACTION OF rFVIIa

Coagulation is initiated by formation of a complex of TF and FVIIa at the site of injury (Fig. 1). TF is a membrane-bound glycoprotein expressed on cells in the subendothelium. Tissue injury disrupts the endothelial

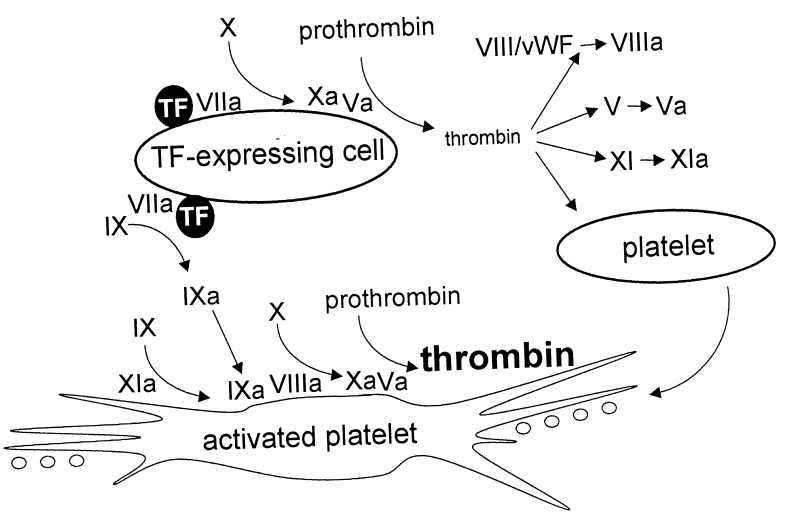

Figure 1 A cell-based model of coagulation (see text for further explanation).

1. **Coagulation is initiated by formation of a complex of TF and FVIIa at the site of injury (Fig. 1).**
Anchor Name: Works at the site of injury *[Agency FCB Halesway Olga Kooi]*

cell barrier that normally separates TF-expressing cells from the circulating blood. Once exposed to the blood, TF serves as a high-affinity receptor for FVIIa. FVIIa alone shows very little proteolytic activity and is only realizing its full enzymatic potential when complexed to TF. The TF-FVIIa complex activates factor X (FX) to factor Xa (FXa), leading to the generation of a small amount of thrombin that activates platelets accumulated at the site of injury.[18] The thrombin formed initially also activates factor XI (FXI) to factor XIa (FXIa), and the cofactors factor V (FV) to factor Va (FVa) and FVIII to factor VIIIa (FVIIIa) in the vicinity of the activated platelets. The activated platelets expose negatively charged phospholipids on their surface and provide the template for further thrombin generation. The TF-FVIIa complex also activates FIX to factor IXa (FIXa). FIXa, FVIIIa, and FVa bind efficiently to the surface of the activated platelet, and further activation of FX occurs via the complex between FIXa and FVI-IIa. The subsequent thrombin burst necessary for formation of a fully stabilized hemostatic fibrin plug mediated by FXa in complex with FVa also occurs on the surface of the activated platelet.

Under normal physiological conditions, roughly 1% of the endogenous factor VII (FVII) circulates as the activated enzyme (FVIIa).[19,20] In vitro experiments with synthetic phospholipid vesicles indicate that at limited concentrations of TF, FVII zymogen may inhibit the activity of FVIIa by competing for binding to TF.[21] High plasma levels of rFVIIa after bolus injection overcome the inhibition by zymogen FVII and ensure that most TF molecules are saturated with rFVIIa, providing maximal activation of the coagulation system at the site of injury. In addition, rFVIIa binds with low affinity to the surface of activated platelets and activates FX independently of TF, thereby generating sufficient thrombin to compensate for the lack of FIXa or FV-IIa.[22] The low-affinity binding of rFVIIa to activated platelets explains the need for superphysiological amounts (pharmacological doses) of rFVIIa. rFVIIa does not bind to resting platelets, which explains why systemic coagulation is not observed after rFVIIa infusion. Rather, the effect is localized to the site of injury, where TF is exposed and platelets are activated.

CHARACTERIZATION OF rFVIIa

Structure of pdFVIIa

Human plasma FVIIa circulates in the blood as a single chain zymogen of 406 residues. Human plasma FVII consists of four discrete domains: an amino terminal (N-terminal) γ-carboxyglutamic acid (Gla) domain, two epidermal growth factor (EGF)–like domains, and a serine protease domain. The active two-chain enzyme is generated by specific cleavage after Arg152 (Fig. 2).[23] The primary site of synthesis and posttranslational modifications of the pdFVII molecule is the liver, and the latter includes (1) γ-carboxylation of 10 glutamic acid residues in the N-terminal part of the molecule, (2) N-glycosylation of asparagine residues in positions 145 and 322, and (3) O-glycosylation of serine residues 52 and 60.

Structure-Function Relationships of FVIIa

The modular organization of FVIIa allows a spatial separation of the events and properties involved in the function of FVIIa. Very simplified, the following summarizes the structure-function relationships of FVIIa: (1) the N-terminal Gla domain binds to phospholipid surfaces, (2) the C-terminal serine protease domain confers the enzymatic activity, (3) the two EGF-like domains are spacers between them, and (4) all four domains contribute to the interaction with TF.

Calcium ions bind to three domains in FVIIa.[24] Without calcium ions FVIIa has virtually no biological activity. Seven calcium sites are located in the Gla domain, and they need to be occupied for FVIIa to bind to cell membranes,[25] such as the surface of the activated thrombocyte,[22] and also for a proper interaction with TF. One calcium ion binds to the first EGF-like domain and the serine protease domain, respectively, influencing TF binding and enzymatic activity.[26,27] Under in vivo conditions, when FVIIa is saturated with calcium ions, the binding to membrane-associated TF dramatically increases the rate by which FVIIa generates FXa (and FIXa).[28] The structural changes in FVIIa upon TF association are few,[29] as suggested from a structural comparison of free and TF-bound FVIIa.[24,30] Nevertheless, the activity of FVIIa is stimulated upon TF binding. This effect is a consequence of the interaction between the protease domain and TF, whereas the other three domains seem primarily to tether FVIIa to TF.[31]

Structure of rFVIIa

The structural characterization of rFVIIa includes determination of the amino acid sequence and the potential posttranslational modifications. The posttranslational modifications of both pdFVIIa and rFVIIa have been investigated and compared.[32] The enzymatic properties of pdFVIIa and rFVIIa have been evaluated and found to be equivalent.[33]

AMINO ACID SEQUENCE

Human pdFVIIa and rFVIIa have been compared by sequence analysis.[32] The light and heavy chains of the two molecules were separated, and peptide mapping of the two chains was carried out followed by amino acid

1. **The TF-FVIIa complex activates factor X (FX) to factor Xa (FXa), leading to the generation of a s...**
Anchor Name: Pure rfVIIa
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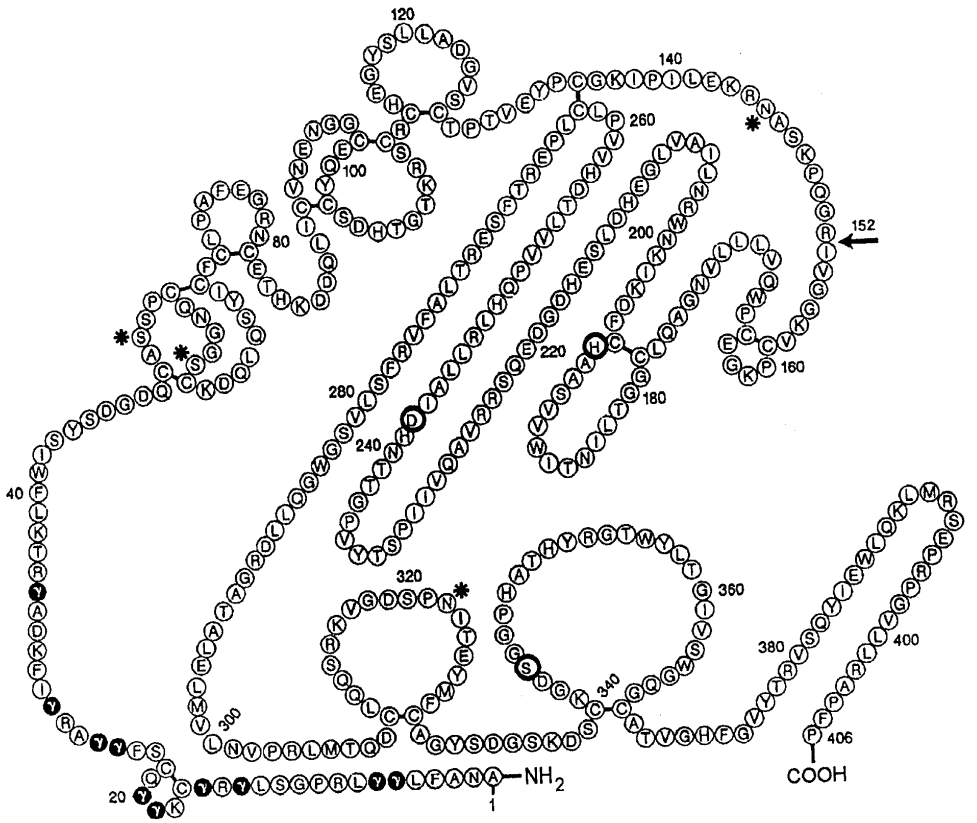

Figure 2 Human coagulation factor VII (FVIIa). FVIIa is a serine protease of 406 residues. The active two-chain enzyme is generated by specific cleavage after Arg152. Posttranslational modifications of the FVII molecule include (1) γ-carboxylation of 10 glutamic acid residues in the N-terminal part of the molecule, (2) N-glycosylation of asparagine residues in positions 145 and 322, and (3) O-glycosylation of serine residues 52 and 60. γ, gamma-carboxyglutamic acid; *, glycosylation sites; →, activation site; O, catalytic site residues.

sequencing of individual peptide fragments. The peptide mapping and sequencing showed that the two molecules were identical with respect to amino acid sequence[32] and that the sequence was identical to that predicted from the cDNA sequence.[23]

γ-CARBOXYLATION

The content of γ-carboxylated glutamic acid residues (Gla) in rFVIIa has been compared with that found in human pdFVIIa.[32] In human pdFVIIa, all 10 possible Gla residues (Fig. 2) were fully γ-carboxylated whereas rFVIIa contained 9 fully γ-carboxylated residues and 1 partially (approximately 50%) γ-carboxylated residue (residue no. 35).[32] Carboxylation of glutamic acid 35 appears to be functionally irrelevant.[34]

β-HYDROXYLATION

The cDNA sequence[23] predicts an aspartic acid residue in position 63 of human pdFVIIa (see Figure 2). By homology to other vitamin K–dependent coagulation proteins, this residue might be β-hydroxylated.[35,36] By the use of a series of different methods, including amino acid analysis, sequencing analysis, and mass spectrometry, it has been shown that neither native pdFVIIa nor rFVIIa contains a β-hydroxy-aspartic acid in position 63.[32]

O-GLYCOSYLATION

Human pdFVIIa contains two O-glycosylated sites: serine 52 and serine 60.[32,37–39] Both O-glycosylation sites were fully occupied in rFVIIa as well as in pdFVIIa. At serine 52, three different glycan structures

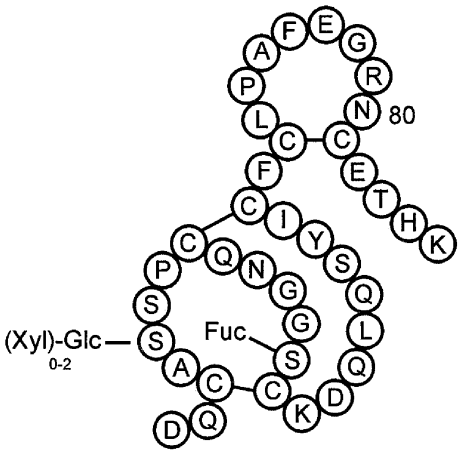

Figure 3 O-glycosylation. Carbohydrate structures O-linked to serine 52 and serine 60 in recombinant factor VII and in plasma factor VII. Glc, glucose; Xyl, xylose; Fuc, fucose.

consisting of glucose, glucose-xylose, or glucose-(xylose)$_2$ were found in both pdFVIIa and rFVIIa (Fig. 3).[37] The relative amounts of the three O-linked structures differed slightly between pdFVIIa and rFVIIa. At serine 60, a single fucose was found in both pdFVIIa and rFVIIa (Fig. 3). In a study of the functional role of the O-linked glycosylation of FVIIa by use of site-specific mutants, it was suggested that the O-glycosylations could provide structural elements that are of importance for the association of FVIIa with TF.[40]

N-GLYCOSYLATION

Human pdFVIIa contains two potential N-glycosylation sites at asparagine 145 and asparagine 322, and both sites were found to be fully occupied in pdFVIIa and rFVIIa. Only minor quantitative differences were seen in the carbohydrate composition of pdFVIIa and rFVIIa, the most pronounced difference being a higher fucose content and a lower sialic acid content of rFVIIa compared with those for pdFVIIa.[32] Later studies have provided a full characterization of the N-linked carbohydrate structures of rFVIIa.[41–44] A total of 15 carbohydrate structures were found, with the major structures being complex biantennary structures with differences in the number of terminal sialic acid residues (Fig. 4).[42] Some of the carbohydrate structures have an N-acetylgalactosamine residue at the position where a galactose is usually found. In general, the same carbohydrates were found at both N-glycosylation sites

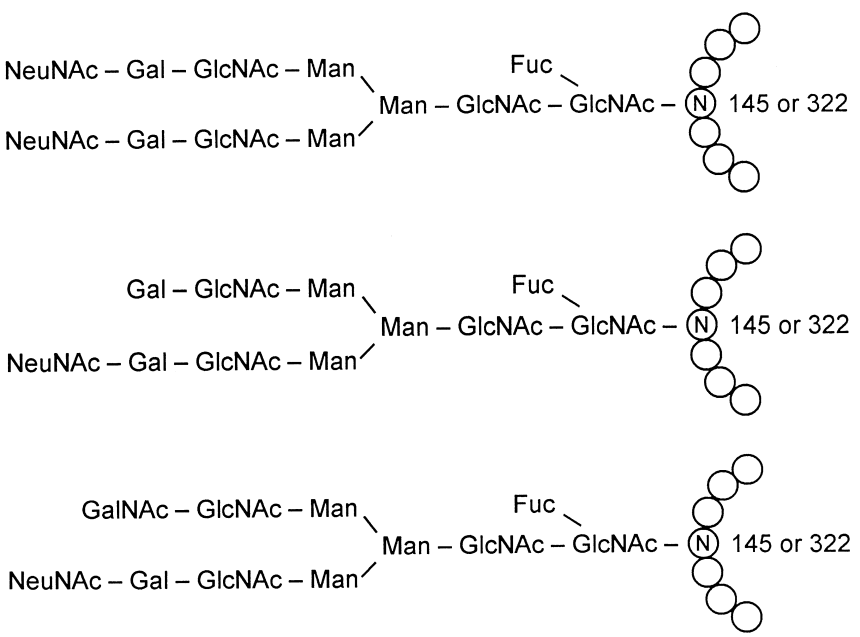

Figure 4 N-glycosylation. The three major carbohydrate structures N-linked to asparagine 145 and asparagine 322 in recombinant factor VII. Fuc, fucose; GlcNAc, N-acetylglucosamine; Man, mannose; Gal, galactose; GalNAc, N-acetylgalactosamine; NeuNAc, N-acetylneuraminic acid (sialic acid).

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of rFVIIa. However, significant differences were found between the two sites in the relative amounts of the carbohydrate structures; for example, the amount of structures with N-acetylgalactosamine was higher at asparagine 322 (30%) compared with its amount at asparagine 145 (7%).[42] Several of the analytic methods used for the carbohydrate characterization have properties in terms of sensitivity and robustness, making them suitable for routine analysis.[41,42] These methods have been used to document reproducible glycosylation of rFVIIa from batch to batch.

MANUFACTURING OF rFVIIa

Expression

The human gene for FVII, located on chromosome 13, is composed of eight exons (coding regions). A cDNA for the coding region of human FVII was isolated from a liver gene library and characterized as previously described by Hagen and coworkers.[23] rFVII was obtained from a mammalian expression system by the use of BHK cells. For details on transfection of BHK cells with human FVII cDNA, see Berkner et al.[45]

A master cell bank (MCB) has been established from the transfected cells and stored in the vapor phase of liquid nitrogen to ensure the stability of the cells during long-term storage. These BHK cells have been thoroughly tested to ensure the presence of a correct gene construct, sterility, and the absence of mycoplasma and viruses. The cells are capable of a stable expression of FVII for several weeks of cultivation and thus suitable for large-scale production.

A working cell bank (WCB) has been created from a single vial of the MCB, and each rFVIIa production run is initiated by thawing one WCB vial. This ensures that the number of cell generations that has elapsed before each step in the process (e.g., inoculation, harvest, termination) is kept constant for different production runs.

The cells are cultivated in a high cell density bioreactor using microcarriers for cell attachment. After a series of propagation steps (Fig. 5), the culture medium derived from the BHK cells containing secreted single-chain rFVII is collected by a draw and fill process and clarified by centrifugation and filtration before purification.

Activation of FVII to FVIIa

From the cloning and sequence analysis of human FVII,[23] it is clear that the activation of FVII to FVIIa involves the specific hydrolysis of a single peptide bond between arginine 152 and isoleucine 153 (see Fig. 2). This activation is probably carried out in vivo predominantly by membrane-bound FXa.[46] In the initial phase of the rFVIIa research project, a series of specific proteases were evaluated. None of the proteases tested could ensure complete cleavage after arginine 152 in rFVII without additional cleavages of other peptide bonds in the molecule. However, during purification of single-chain FVII by ion-exchange chromatography, it was observed that part of FVII was converted into the activated two-chain form.[47] Subsequent sequence analysis showed that the two-chain form was identical to pdFVIIa, in other words, specific hydrolysis had occurred between residues 152 and 153. When this process was optimized, rFVII was converted into rFVIIa with almost 100% yield.[32,47] The mechanism of activation is still not fully elucidated. The degree of activation seems to depend on the amount of rFVII loaded per volume of ion-exchange material. Single-chain rFVII has no proteolytic activity by itself,[48] but trace amounts of rFVIIa could be generated by cellular proteases or proteases released to the medium, and this rFVIIa could initiate the autoactivation process when rFVII and rFVIIa is concentrated on the ion-exchange column.

Purification

The purification method for rFVII from the cell culture medium has to ensure the removal of non-rFVII protein and the specific activation of rFVII to rFVIIa. The following purification procedure (see Figure 5) was developed to meet these criteria: (1) culture medium is pH adjusted and loaded onto a Q-Sepharose FF® (Amersham Pharmacia Biotech, Hørsholm, Denmark) column. This step mainly functions to concentrate the protein. (2) Virus inactivation is ensured by treatment with a detergent. (3) rFVII is then loaded on an immunoaffinity column.[32] This step very efficiently purifies rFVII because both the binding of rFVII to the column and the elution of rFVII from the column is specific for the rFVII protein. (4) The final purification and the complete activation of rFVII to rFVIIa is carried out by the use of two anion-exchange chromatography steps. The purified and activated rFVIIa bulk drug substance is formulated into a solution of the composition given in Table 1. This solution is dispensed into vials and freeze dried. The final drug product, NovoSeven®, exists in three presentations containing 1.2 mg, 2.4 mg, and 4.8 mg of rFVIIa, respectively.

PRECLINICAL DEVELOPMENT

Preclinical Efficacy and Safety

The hemostatic effect of rFVIIa has been verified in hemophilic dogs and by warfarin-induced bleeding in rats. rFVIIa (dose range 50 to 220 μg/kg body weight [b.w.]) was able to correct the cuticle bleeding time in dogs with hemophilia A or B.[49] In cases of warfarin-

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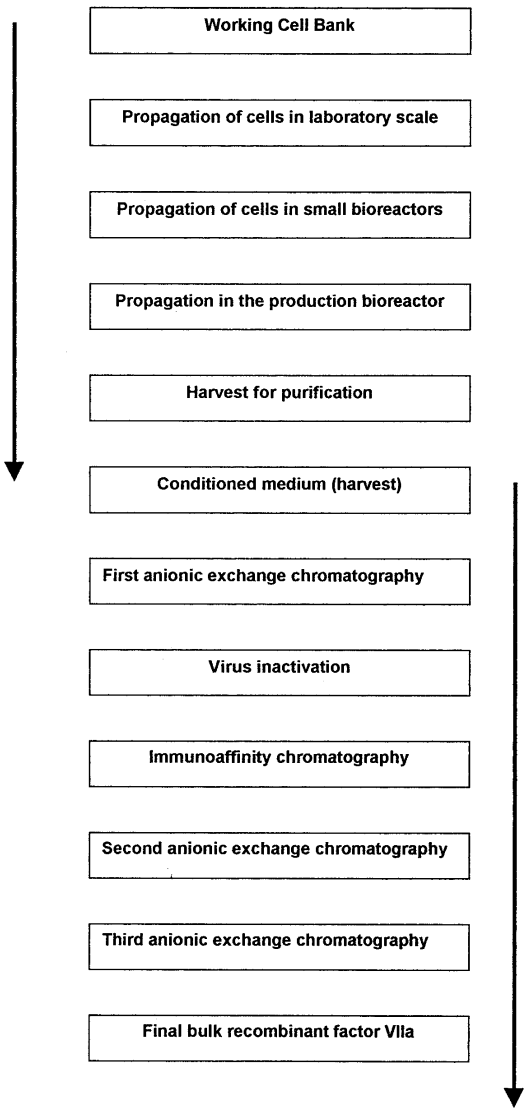

Figure 5 Manufacturing of rFVIIa. A WCB has been created from a MCB and is cultivated in a high cell density bioreactor using microcarriers for cell attachment. After a series of propagation steps the culture medium is collected by a draw and fill process and clarified by centrifugation and filtration before purification. During the purification process single-chain rFVII is autoactivated into the two-chain form during the final anion-exchange chromatography.

induced bleeding in rats, the bleeding from the tail cuts was partially normalized by 50 μg/kg b.w. rFVIIa and fully normalized by 250 μg/kg b.w. rFVIIa.[50] However, it should be emphasized that because of the species difference with regard to the binding between TF and FVIIa, no conclusions on optimal doses in humans can be drawn from these experiments.

The theoretical lack of systemic activation after rFVIIa treatment has been supported by preclinical data. Studies in the standard rabbit stasis model, developed as a thrombosis model in which injury was induced to the vessel wall, have demonstrated that rFVIIa (100 to 1000 μg/kg b.w.) or prothrombin complex concentrate (FEIBA; 50 to 100 U/kg; Immuno, Deerfield,

Table 1 Final Formulation of Product before Filling and Freeze Drying

rFVIIa	0.6 mg/mL
NaCl (50 mM)	2.92 mg/mL
Calcium dichloride dihydrate (10 mM)	1.47 mg/mL
Glycylglycine (10 mM)	1.32 mg/mL
Tween 80	0.07 mg/mL
Mannitol	30 mg/mL

IL) caused clot formation at the site of injury after 30 minutes of stasis (restricted blood flow). This reflects the normal pharmacological response to tissue injury. Three hours after administration, rFVIIa caused no change in platelet count or fibrinogen concentration. Furthermore, no changes were noted in antithrombin levels, nor was there any evidence of generation of soluble fibrin monomers, as judged by an ethanol gelation test. In contrast, FEIBA caused a significant dose-dependent decrease in platelets and fibrinogen, suggesting a general activation of the coagulation system. Administration of rFVIIa (100 to 300 μg/kg) to rabbits previously exposed to endotoxin did also not result in any significant hematologic changes (i.e., decreased leukocyte count, platelet count, or fibrin monomers), compared with rabbits treated with endotoxin alone.[51]

CLINICAL DEVELOPMENT

Pharmacokinetics in Patients

rFVIIa pharmacokinetics has been studied in adult and pediatric hemophilia patients, as well as in adults with acquired FVII deficiency (i.e., healthy adult volunteers pretreated with acenocoumarol and patients with liver cirrhosis).[52–56] The clearance and half-life values (range 2.4 to 3.2 hours) of rFVIIa after bolus administration were in the same range in the adult populations studied. Pediatric patients with hemophilia had a shorter half-life (1.3 hours) and a higher clearance than the adults with hemophilia had.[54,56]

Efficacy Trials in Congenital or Acquired Hemophilia Patients with Inhibitors

Since 1988, the therapeutic efficacy and safety of rFVIIa in hemophilia patients with inhibitors have been investigated in a number of international clinical studies. The largest trial to date is the Compassionate Use Study (n = 148),[5–7,11] including hemophilia A and B patients, acquired hemophilia patients, and FVII-deficient patients. The purpose of the program was to treat patients with life- or limb-threatening bleeds when all other therapeutic alternatives had been exhausted. In the Dose Finding Study[3] (n = 78), a randomized, double-blind, multicenter study including hemophilia patients with inhibitors and joint, muscle, and mucocutaneous bleeding episodes were treated with rFVIIa (35 or 70 μg/kg). Another randomized, double-blind, multicenter, dose-finding study, the Surgery Study[2] (n = 29), included hemophilia patients with inhibitors who had been scheduled for elective minor or major surgery. Finally, a multicenter Home Treatment Study[1] (n = 60) of hemophilia patients with inhibitors documented and emphasized the benefits of early initiation of therapy with rFVIIa. All studies showed that treatment with rFVIIa is effective (80 to 90%) and safe in the dose range of 35 to 120 μg/kg, with a recommended dose of 90 μg/kg to be given as a bolus dose and repeated after 2 hours. When more than two doses are necessary to ensure and maintain hemostasis for minor or moderate bleeds, the dose-interval may be prolonged from 2 to 6 hours, depending on the size and severity of the bleed. Successful surgical treatment regimens and treatment of major life- or limb-threatening bleeds, however, necessitates administration of rFVIIa every 2nd hour for the first 24 hours, and then the dose interval may increase over the next 3 days from 2 to 6 hours depending on the type of surgery performed.[15,16] The level of the inhibitor to FVIII or FIX does not influence the efficacy of rFVIIa, nor does rFVIIa evoke an anamnestic response in FVIII- or FIX-deficient patients.[57] Therefore, rFVIIa is a suitable treatment for acute bleeding episodes or control of hemostasis during surgery in hemophilia patients with inhibitors and prior to or during initiation of immune tolerance therapy.[58–60]

Clinical Safety

Since the first launch (July 1996), more than 180,000 standard doses of rFVIIa have been administered. The frequency of spontaneously reported serious adverse events per treatment episode is 0.6%. The incidence of thrombotic events is less than 0.4%, and 50% of the thrombotic events occurred in patients more than 65 years of age.

Clinical Data Outside Hemophilia

Case stories have been published on the use of rFVIIa outside hemophilia, including patients with known other abnormalities of the coagulation system, in other words, patients with other congenital or acquired coagulation deficiencies[12,61–63] or platelet defects[64–67] and patients *without* known abnormalities of the coagulation system but with life- or limb-threatening bleeds, for which all other therapeutic alternatives had been exhausted or no other treatment options currently existed (i.e., trauma patients, acute upper gastrointestinal

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bleeding, intracerebral hemorrhage patients, bleeding associated with renal failure).[68–73]

In FVII-deficient patients (n = 17) successful treatment of 27 spontaneous bleeding episodes and 7 major and 13 minor surgical interventions were reported using a mean dose between 22 and 26 μg/kg b.w.[12] Other reports have confirmed that rFVIIa may be a valuable substitution therapy in severe hereditary FVII deficiency in doses of 20 to 30 μg/kg b.w.[13,14]

In acquired FVII deficiency associated with vitamin K treatment or coagulopathy due to chronic liver disease, or both, clinical randomized studies in patients treated with acenocoumarol (n = 28) or with liver cirrhosis (n = 10) have shown that the prolonged prothombin time can be reduced by the use of rFVIIa.[52,61,63]

Patients with quantitative or qualitative platelet disorders (i.e., thrombocytopenia, Glanzmann's thrombasthenia, or Bernard-Soulier syndrome) have been reported to be successfully treated with rFVIIa. In thrombocytopenia patients, bleeding time was reduced in 55 episodes of 105 infusions of rFVIIa.[64–66] In patients with bleeding during their thrombocytopenia, the bleeding stopped in 6 out of 8 patients.[64] In Glanzmann's thrombasthenia, a total of 24 bleeding episodes, including 4 episodes in children, which also included a surgical intervention, were reported to be successfully treated with rFVIIa in dosages of 89 to 116 μg/kg b.w. per injection every 2 hours.[66]

While awaiting results from prospective randomized trials, data from patients being treated experimentally with rFVIIa outside the current indications are being entered and monitored in newly established clinical registries (http://www.haemophilia-forum.org/ and http://www.novoseven.com).

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