

Coagulation factor VII and follicle stimulating hormone. Made by genetic engineering (No. 8 in a series of articles to promote a better understanding of the use of genetic engineering)

NovoSeven®: enhancing the natural coagulation pathway in haemophiliacs with inhibitors

NovoSeven® is an activated human coagulation factor VII made by recombinant technology (Table 1). Factor VII is one of the key molecules that takes part in the initiation of the coagulation cascade. The molecule is able to directly activate factor X and thereby initiate the generation of thrombin independently of the so-called amplification cascade including FVIII and FIX (Fig. 1). In this way NovoSeven® can initiate coagulation in patients suffering from FVIII deficiency (haemophilia A) and FIX deficiency (haemophilia B). Most importantly, NovoSeven® can also initiate coagulation in the sub-group of these patients suffering from inhibitors towards coagulation factors FVIII and FIX. Until now, satisfactory treatment of these patients has not been possible.

NovoSeven® is a complex glycoprotein of 406 amino acids with 2 *N*- and 2 *O*-glycosylation sites. The *O*-glycosylation sites are in the epidermal growth factor region of the molecule attached to Ser52 and Ser60, while the *N*-glycosylation sites are Asn145 and Asn322. The *O*-glycosylation sites consist of one to four monosaccharides, while the *N*-glycosylations mainly are biantennary core-fucosylated with one or two sialic acid residues. In the N-terminal, 10 γ -carboxylic acid residues have been identified. All these post-translational modifications are of importance for the

biological function of the molecule. Therefore, a mammalian cell fermentation system is needed for the biotechnological production of FVII. As a baby hamster cell line has the capacity to γ -carboxylate heterologous molecules, this cell line has been selected. These cells secrete rFVII (recombinant FVII) to the culture medium. During the purification rFVII is spontaneously auto-activated in vitro.

The cells are grown in large stainless steel fermenters in a continuous process. No human proteins are added, neither during the fermentation, purification nor final formulation. The final product contains only one protein, NovoSeven®. All impurities measurable by ELISA methods are in the ppm range.

The plasma derived product and the recombinant product are identical except for minor differences in the *N*-glycosylation. The glutamic acid residue in position 35 is only partially carboxylated in the recombinant product, but the biological potency is the same as for plasma derived FVII.

1. Clinical

Normal haemostasis is initiated by the formation of a complex between a so-called tissue factor (a receptor lipoprotein exposed at the site of injury to the vessel wall and activated FVII/FVIIa) in the circulating blood. This complex

Coagulation Cascade

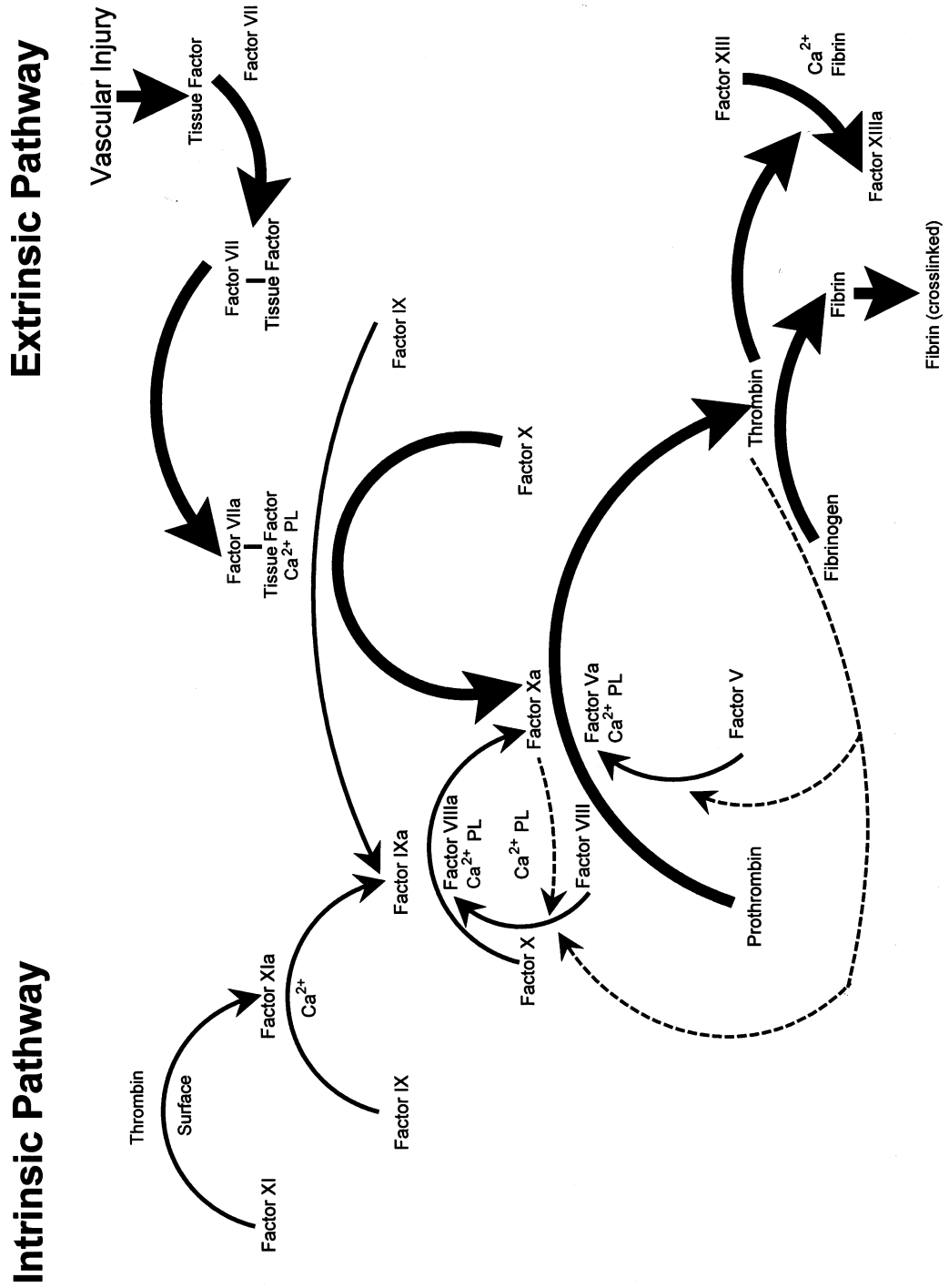


Fig. 1.

activates FX and FIX of the coagulation cascade locally at the site of the complex on the injured vessel wall providing the first molecules of thrombin from prothrombin. Thrombin converts fibrinogen into fibrin thereby forming a haemostatic plug. Thrombin also activates platelets as well as FVIII and FV, both co-factors active in the further coagulation. While FVIIa-TF (thrombin/fibrin) is important for initiation of haemostasis, FIX and FVIII maintain haemostasis after the FVIIa-TF-pathway has been inhibited by the tissue factor pathway inhibitor (TFPI). By stimulating the normal initiation of haemostasis (adding exogenous FVIIa) bleeding defects characterized by impaired function of other parts of the coagulation mechanism can be compensated for.

Recombinant FVIIa has a haemostatic efficiency of between 76 and 94% in 422 patients with haemophilia A ($n = 339$), haemophilia B ($n = 39$) and nonhaemophiliacs with antibodies against FVIII ($n = 44$), all having antibodies against FVIII or FIX. Most of the patients were treated in association with life-threatening and limb-damaging bleeds after conventional therapy (coagulation factor concentrates, activated or non-activated prothrombin complex concentrates, porcine FVIII) had failed. Therefore, most patients were in extremely serious conditions. In spite of this an efficacy rate of 85% was achieved in stopping bleeding in the central nervous system. All patients recovered without any sequelae, while the mortality in CNS bleeds in uncomplicated haemophilia (no antibodies) is c. 50%. In major surgery including orthopaedic surgery neither per- nor post-operative bleeding was observed. Joint bleeds were stopped with an efficacy rate of 91% when given in a home-treatment setting when patients are able to initiate the treatment immediately at the debut of any symptom; rFVIIa treatment in muscle bleeds had an efficacy of 94%. The efficacy of rFVIIa in stopping bleeding in haemophiliacs with inhibitors against FVIII/FIX was thus as high as reported for recombinant FVIII in FVIII-deficient patients (haemophilia A) that did not suffer from complications due to antibodies against FVIII.

Many patients were given rFVIIa several times

(72 more than ten times). No antibody formation against rFVIIa has been found in these patients. Very few side-effects possibly related to rFVIIa have been observed.

2. Conclusion

Recombinant FVIIa is being produced in BHK cells and is hemostatically effective in c. 87% of serious bleeds and surgery in patients with inhibitors against coagulation factors VIII or IX.

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Infertility treatment by recombinant FSH

Follicle stimulating hormone (FSH) is a heterodimeric glycoprotein produced in the anterior pituitary gland. The hormone is essential in the regulation of the reproductive processes, such as follicular development and ovulation. It is clinically used for treatment of anovulation and in assisted reproduction technologies (ARTs) such as in vitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI).

Until very recently, the only source of human FSH was the urine of post-menopausal women, in which the hormone is present in relatively high concentrations. However, the use of natural sources has a number of drawbacks, including potential product variability and a limited availability. As a result of improved diagnostic, therapeutic and monitoring procedures the world-wide demand for FSH is steadily increasing, which has already led to shortages in FSH supply.

In the early 1980s Organon decided to develop an alternative production route of human FSH via recombinant DNA technology (Table 1). FSH (like thyroid stimulating hormone, luteinizing hormone (LH) and human chorionic gonadotropin) belongs to a family of glycoproteins

that are composed of two dissimilar subunits named α and β , which are associated by non-covalent bonds. The first challenge in the project was the cloning of the two FSH genes. The α -subunit is common to all four glycoprotein hormones and is encoded by a single gene, whose complete nucleotide sequence had already been published. Because the complete gene was too large to manipulate, parts of the non-coding sequence were omitted and a smaller hybrid gene was constructed. The β -subunits of the glycoprotein family are encoded by different genes and determine the biological activity of the individual hormones. In the early 1980s no reports were available on the nucleotide sequence of the human β -FSH gene. The published amino acid sequence of the β -subunit of FSH was used to generate synthetic oligonucleotides for screening of cDNA and genomic libraries. Finally, this resulted in the isolation of the complete β -FSH gene.

The production of FSH was the second challenge, because of the need for proper assembly of the α - and β -subunits and post-translational modifications of the amino acid backbone. Both the α - and β -subunit of FSH contain two *N*-linked oligosaccharide side chains. These carbohydrates serve many important functions. They are required for proper folding, assembly and secretion of FSH and are also highly relevant for its *in vivo* biological activity. It is well established that glycosylation determines the half life of the glycoprotein hormones. Thus, proper post-translational modification is an absolute requirement for full biological activity of FSH.

Depending on the host-cell type a large repertoire of FSH molecules is produced, in which each variant species (glycoform) differs somewhat in the composition of the oligosaccharide side chains. Initially, the sugar residues are attached to the amino acid backbone in the endoplasmic reticulum. Further processing involves trimming by glucosidases and mannosidases, followed by remodelling of the carbohydrates in a complex series of biochemical reactions. This complex series of reactions can only be performed properly by mammalian cells.

The Chinese hamster ovary (CHO) K1 cell line—which had been initiated in 1957 from the ovarian biopsy of an adult hamster—was selected as the ‘protein factory’ for the production of the recombinant FSH. This cell line proved to be easily

transfected, with the assurance of proper glycoprotein expression and safety. Analyses showed that the selected CHO clone was genetically stable and capable of secreting biological active recombinant human FSH (rhFSH) for a prolonged period of time. A production process for rhFSH was developed. After careful purification, a product which is very similar to the authentic human FSH was obtained.

As a result of the extensive biochemical processing each of the oligosaccharide side chains demonstrate considerable microheterogeneity. Typically, this microheterogeneity causes charge heterogeneity due to differences in the amount of terminal sialic acid residues. Isoelectric focusing and chromatofocusing are widely employed to visualise microheterogeneity. The majority of the rhFSH molecules has an isoelectric point between pH 4.3–5.3. This range is comparable to that of FSH from human pituitary origin and slightly less acidic than FSH from urinary origin (Metrodin®).

A large prospective, randomised, assessor-blind, multicentre study was performed to compare recombinant- and urinary human FSH in IVF. The results indicate that recombinant FSH is more efficacious than urinary FSH as assessed by the number of oocytes retrieved and ongoing pregnancy rate including frozen embryo cycles. In May 1996 Organon’s recombinant human FSH was registered in the European Union under the trade name Puregon®. What would currently require millions of litres of urine per year, can now be produced by genetically engineered cells in chemically defined culture media comprising only a small fraction of that volume. Complex issues related to the availability, variability and nature of the raw material (human urine) can now be eliminated. The DNA technology has provided an inexhaustible supply of pure human FSH, which is free of LH and offers the advantages of better batch-to-batch consistency, greater purity and the absence of human contaminants.

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The products published in this series so far are summarised in Table 2.

Table 1
Made by genetic engineering No. 8

Product	Human coagulation factor VII	Follicle stimulating hormone (FSH)
Principal trade names	NovoSeven®	Puregon®
Principal uses	Human therapeutical	Treatment of anovulation, use in assisted reproduction technologies
Manufacturer	Novo Nordisk A/S	N.V. Organon, Akzo Nobel
Donor organism	Human gene	Human gene
Host organism	Baby hamster kidney cells	Chinese hamster ovary cells
Advantages	<ul style="list-style-type: none"> –Haemostatic effect in haemophilia independent of FVIII and FIX –No thromboembolic side effects –No risk of transfer of human blood-borne viruses 	<ul style="list-style-type: none"> –Efficient production in chemically defined culture media guarantees inexhaustible supply –Better batch-to-batch consistency –Greater purity, free of luteinizing hormone (LH), absence of human contaminants

Table 2
Summary of products published in the series made by genetic engineering

Product:	Lipase	Hepatitis B vaccine
Author/Manufacturers	B. Diderichsen, Novo Nordisk, Denmark	P. Crooy, SmithKline Beecham Biologicals, Belgium
Made by Genetic Engineering No. 1	<i>Biotech Forum Europe 8, 246–247, 1991</i>	
Product:	Human insulin	Human growth hormone
Author/Manufacturers	E. Rasmussen, Novo Nordisk, Denmark	L. Fryklund, KabiPharmacia, Sweden
Made by Genetic Engineering No. 2	<i>Biotech Forum Europe 9, 144–145, 1992</i>	
Product:	Protein G	Interferon alfa-2a
Author/Manufacturers	R. Hjorth, Pharmacia LKB Biotechnology, Sweden	S. Ryser, F. Hoffmann-La Roche AG, Switzerland
Made by Genetic Engineering No. 3	<i>Biotech Forum Europe 9, 641–642, 1992</i>	
Product:	AIDS test	α-Amylase
Author/Manufacturers	E. Baumann, F. Hoffman-La Roche AG, Switzerland	B. Diderichsen, Novo Nordisk, Denmark
Made by Genetic Engineering No. 4	<i>J. Biotechnol. 38, 193–197, 1995</i>	
Product:	Erythropoietin	Interferon beta-1b
Author/Manufacturers	C. Kionka, Boehringer Mannheim, Germany	T. Petri, Schering AG, Germany
Made by Genetic Engineering No. 5	<i>J. Biotechnol. 43, 73–77, 1995</i>	
Product:	Interferon gamma	Rabies vaccine
Author/Manufacturers	E. Falkner and I. Maurer-Fogy, Bender & Co GesmbH/Boehringer Ingelheim Vienna, Austria	J. Terré, G. Chappuis, M. Lombard and P. Desmetre, Rhône Mérieux, France
Made by Genetic Engineering No. 6	<i>J. Biotechnol. 46, 155–159, 1996</i>	

Table 2 (continued).

Product:	Tissue plasminogen activator (rt-PA)	Granulocyte-macrophage colony-stimulating factor (GM-CSF)
Author/Manufacturers	W. Werz and R.G. Werner, Dr Karl Thomae GmbH/Boehringer Ingelheim Pharma, Germany	R. Till, Novartis/Schering Plough International, Switzerland
<i>Made by Genetic Engineering No. 7</i>	<i>J. Biotechnol., 157–161, 1998</i>	
Product:	Human coagulation factor VII	Folicle stimulating hormone (FSH)
Author/Manufacturers	Novo Nordisk A/S	N.V. Organon, Akzo Nobel
<i>Made by Genetic Engineering No. 8</i>	<i>J. Biotechnol., 231–236, 1998</i>	
