Production of Recombinant Blood Clotting Factors in Human Cell Lines

Introduction

The present invention relates to an improved method for the production of recombinant human blood clotting factors, in particular of factor VIII and factor IX, utilizing an immortalized human cell line stably expressing viral transcription activator proteins and carrying a vector having a promoter functionally linked to a DNA sequence coding for a blood coagulating factor, provided that said promoter is not a viral promoter which is stimulated by said viral transcription activator proteins; an immortalized human cell line carrying said vector; factor VIII muteins particularly suitable for the above production method; pharmaceutical compositions comprising such factor VIII muteins and the use of such factor VIII muteins for preparing a medicament for treating hemophilia.

Summary of the Related Art

Hemophiliacs are suffering from hemorrhagic morbidity caused by the disturbed function of protein components of the blood coagulation cascade. Dependent on the affected clotting factor two types of hemophilia can be distinguished. Both have in common the inhibited conversion of soluble brinogen to an insoluble fibrin-clot. They are recessive X-chromosomally-linked genetic diseases affecting mainly the male population.

Hemophilia A affects 1-2 individuals per 10.000 males. It is caused by the deficiency or absence of factor VIII, a very large glycoprotein (Mr approximately 330 kDa (Furie B., Furie B.C., *Cell* (1988) 53, 505-518)), which represents an important element of the blood coagulation cascade. The polypeptide sequence can be subdivided in three regions, an N-

terminal region consisting of the so-called A1 and A2-domains, a central B-domain region and a C-terminal region composed of the A3, C1 and C2 domains. In the blood coagulation factor VIII occurs as an inactive precursor. It is bound tightly and non-covalently to von Willebrand Factor (vWF), which acts as a stabilizing carrier protein. Proteolytical cleavage of factor VIII by thrombin at three specific positions (740, 372, 1689) leads to its dissociation from vWF and releases the procoagulant function within the cascade. In its active form factor VIII functions as a cofactor for factor IXa, thereby accelerating the proteolytic activation of factor X by several orders of magnitude.

Hemophilia B occurs in about 1 of 25,000 males. It is characterized by the deficiency of the serine protease factor IX (Christmas factor). This 415 amino-acid polypeptide is synthesized in the liver as a 56kDa glycoprotein. In order to attain its proper function a posttranslational carboxylation step is required which only occurs in the presence of vitamin K.

Treatment of both types of bleeding disorder traditionally involves infusions of human plasma-derived protein concentrates of factor VIII or factor IX. Although this method represents an efficient therapy for hemophiliacs it carries the risk of transmission of various infectious agents, such as viruses causing hepatitis or AIDS, or thromboembolic factors. Alternatively several recombinant DNA techniques for the production of clotting factors have been described. For this purpose the corresponding cDNAs of wild type factor VIII and factor IX have been isolated and cloned into suitable expression vectors (EP-A-160457; WO-A-86/01961, U.S. Patents 4,770,999, 5,521,070 and 5,521,070).

In the case of factor VIII recombinant expression of subunits for the production of complexes showing coagulant activity is known in the art (e.g., from EP-A-150735, EP-A-232112, EP-A-0500734, WO-91/07490, WO-95/13300 U.S. Patents 5,045,455 and 5,789,203). Moreover, the expression of truncated cDNA-versions partially or entirely lacking the sequence

coding for the highly glycosylated B-domain have been described (e.g. in WO-86/06101, WO-87/04187, WO-87/07144, WO-88/00381, EP-A-251843, EP-A-253455, EP-A-254076, U.S. Patents 4,868,112 and 4,980,456, EP-A-294910, EP-A-265778, EP-A-303540 and WO-91/09122). More recently a variety of selected point mutations have been introduced to inhibit proteolytic inactivation of factor VIII by activated protein C or to reduce the immunogenicity resulting in the formation of inhibitory antibodies by the treated patients (e.g., U.S. Patents 5,859,204, 5,422,260 and 5,451,521, WO-97/49725 and WO-99/29848).

The recombinant clotting factors were usually isolated from the medium of stably transfected eukaryotic and preferably mammalian cell lines. It was, however, general practice to employ non-human cell lines in the production methods disclosed in the references mentioned herein before in order to exclude the risk of copurifying some infectious agents which may be harbored and expressed by human cells.

However, especially for factor VIII, the use of non-human cell lines encountered certain disadvantages. For example unsatisfactory secretion levels of the expressed protein into the medium has been reported. This may be due to slight differences within different types of mammalian cells concerning intracellular pathways for protein translation and modification, which also might have an effect on the biological activity of the expressed polypeptide. Apart from this, there were concerns that the therapeutic proteins purified from non-human expression systems are contaminated with cellular components which can give rise to antigenic reactions in the patients.

Moreover, proteins expressed by non-human expression systems may have non-human glycosylation patterns giving rise to antigenic reactions in the patient. However, biological stability and efficacy of clotting factors is substantially influenced by their N-glycosylation pattern. Especially

peripheral and terminal monosaccharides are important, because they are detected by specific receptors from cells which are responsible for their degradation. Clotting factors carry as terminal monosaccharides sialic acid residues. Modification in the composition of sialic acids in the antennae of glycoproteins as for example the clotting factors can result in heterogenous glycosylation patterns. Thus, biological stability and efficacy is crucially involved when modification occurs. Hence, it is an important consideration in the production of recombinant clotting factors to evaluate the influence of glycosylation from non-human production cell lines versus human cell lines. Generally spoken, it seems plausible that human cell lines are more qualified for the production of recombinant clotting factors than non-human. The reason for this assumption is that probably no extraneous oligosaccharide will be incorporated into the oligosaccharide moiety during synthesis of recombinant factors.

On the other hand, general methods for high level protein expression of a desired gene comprising immortalized, stably transfected mammalian cell lines expressing viral transcription activator proteins have been made available for some time (e.g. U.S. Patent 5,712,119). Further these cell lines are transformed with a vector construct where a suitable viral transcription promoter is operatively associated with a DNA sequence defining a gene of interest, the transcription activator proteins activate the viral transcription promoter and hence initiate the expression of the gene of interest. Again, there were concerns that the transcription activator proteins expressed by these cell lines may give rise to contaminations in the target therapeutic protein.

In view of the above there was still a need for an effective production method for human blood clotting factors.

Surprisingly, it was found that a non-contaminated blood clotting factor can be obtained with the above mentioned imortalized human cell lines. In

particular, the immortalized cell lines - if carrying a vector having a promoter functionally linked to a DNA sequence coding for the blood clotting factor and despite the fact that the promoter is not a viral promoter which is stimulated by said viral transcription activator proteins - are capable of expressing the blood clotting factor. In combination with suitable protein purification and virus-inactivation protocols this method provides an effective system to produce safe and highly active recombinant blood clotting factors for therapeutic applications in humans. Moreover, particular factor VIII muteins were found which are exceptionally stable against proteolytic inactivation and thus allow to be subjected to vigorous virus inactivation protocols.

Summary of the Invention

The present invention provides

(1) a method for the production of recombinant human blood clotting factor which comprises

(a) culturing an immortalized human cell line stably expressing at least one viral transcription activator protein and carrying a vector having a promoter functionally linked to a DNA sequence coding for the human blood clotting factor, provided that said promoter is not a viral promoter which is stimulated by said at least one viral transcription activator protein, and

(b) isolating the blood clotting factor from the culture broth;

(2) a preferred embodiment of the method defined in (1) above, wherein the human blood clotting factor is factor VIII or a mutein thereof;

(3) a preferred embodiment of the method defined in (2) above, wherein the factor VIII is a mutein having at least one of the following mutations:

(a) Val at position 162 has been replaced by another neutral amino acid residue,

(b) Ser at position 2011 has been replaced by another hydrophilic amino acid residue,

(c) Val at position 2223 has been replaced by an acidic amino acid residue, and

(d) the B-domain between positions Arg740 and Glu1649 has been replaced by an Arg-rich linker peptide comprising 10 to 25, preferably 14 to 20 amino acid residues, wherein said factor VIII numbering is relative to the mature wild-type factor VIII sequence shown in SEQ ID NO: 2;

(4) a preferred embodiment of the method defined in (1) above, wherein the human blood clotting factor is factor IX or a mutein thereof;

(5) an immortalized human cell line carrying a vector coding for a human blood clotting factor as defined in (1) to (4) above;

(6) a factor VIII mutein as defined in (3) above;

(7) a DNA sequence coding for the factor VIII mutein as defined in (6) above;

(8) a vector comprising the DNA as defined in (7) above;

(9) a vector as defined in (8) above which is a gene transfer vector;

(10) a host cell being transformed with a vector as defined in (8) above and/or comprising a DNA sequence as defined in (7) above ;

(11) a pharmaceutical composition comprising the factor VIII mutein as defined in (6) above or a gene transfer vector as defined in (9) above;

(12) the use of the factor VIII mutein as defined in (6) above or a gene transfer vector as defined in (9) above for preparing a medicament for treating hemophilia; and

(13) a method for treating hemophilia which comprises administering human hemophiliacs a factor VIII mutein as defined in (6) above or a gene transfer vector as defined in (9) above.

Description of the figures

Fig. 1 shows the fragments utilized for the construction of factor VIII with a deleted B-domain (Example 1).

Fig. 2 shows the vector pTGF8-1, 8720 bps circular DNA, the exact DNA sequence thereof is given in SEQ ID NO: 3 (for the factor VIII protein encoded by said DNA sequence see SEQ ID NO: 4).

Fig. 3 shows vector pTGFG36, 5753 bps circular DNA, the exact DNA sequence thereof is given in SEQ ID NO: 6 (bases 689-2071 within SEQ ID NO: 6 coding for the factor IX protein).

Fig. 4 shows vector pTG36hyg, 8124 bps circular DNA.

Fig. 5A depicts a preferred linker sequence of the present invention (SEQ ID NO: 9).

Fig. 5B shows the coagulation time of recombinant hFVIII as determined in Example 6.

Fig. 6 shows the common molecular structure of pTGF8-2hyg-s and pTGF8-3, 10698 bps circular DNA, the exact DNA sequences thereof are given in SEQ ID NOs: 12 and 14 (for the factor VIII protein encoded by said DNA sequence see SEQ ID NO: 13 and 15).

Fig. 7 A shows the calibration curve of FVIII ELISA as described in Example 5.

Fig. 7 B depicts the results of the determination of recombinant FVIII concentrations in different culture filtrates as described in Example 5.

Fig. 8 shows the results of a factor VIII specific immunofluorescence assay as described in example 9. Upper row: 293T cells stably transfected with pTGF8-3, clone 49/19. Lower row: Negative control: Untransfected 293T cells. A and C: white light, no filter; B and D: Factor VIII detection by fluorescence, filter 550 nm.

Fig. 9 shows the influence of thermal treatment on FIX activity in culture filtrate as described in example 10.

Fig. 10 shows the dependence of expression of active recombinant factor IX on the supplementation of vitamin K into culture medium.

Detailed Description of the Invention

"Functionally linked" refers to configurations of the vector where the promoter is located within the vector in such a manner that it can stimulate transcription of the DNA sequence coding for the human blood clotting factor. "Not functionally linked" refers to a configuration where the promoter is so remotely located from the expressed gene sequence of the blood clotting factor that it cannot stimulate its transcription.

"Gene" refers to a DNA sequence encoding a polypeptide optionally including leader and trailer sequences and introns and exons.

"Vector" refers to any genetic construct, such as plasmid, phage, cosmid, etc., which is capable of replication when associated with the proper control elements. The term includes cloning and expression vehicles. "Carrying a vector" includes both, the stable and transient incorporation of a functional DNA segments into the host cell. The stable incorporation is, however, preferred.

"Gene transfer vector" in accordance with the present invention includes a vector suitable for gene therapy. Such vector comprises functional sequences for the desired purpose as known in the art.

The term "mature" refers to the molecular structure of a given protein directly after its cellular secretion (i.e., lacking its N-terminal export-signal polypeptide).

"Promoter" refers to a region of regulatory DNA sequences for the control of transcription of a gene to which RNA polymerases bind.

"Therapeutically effective dose" of the pharmaceutical composition of the invention refers to a dose effective for treatment or prophylaxis, for example, a dose that yields effective treatment or reduction of the symptoms of hemophilia. The determination of a therapeutically effective dose is within the purview of one skilled in the art.

"Encodes" or "encoding" refers to a property of the nucleic acid sequence being transcribed (in case of DNA) or translated (in case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of an appropriate regulatory sequence.

For the purpose of this application "express", "expressing" or "expression" refers to the transcription and translation of a gene encoding a protein.

The present invention as described in (1) to (13) above is hereinafter described in more detail. In accordance with embodiment (1) of the invention of the present application the promoter functionally linked to the DNA sequence coding for the human blood clotting factor is not a viral promoter which is stimulated by the at least one viral transcription activator protein expressed by the immortalized human cell line.

The immortalized human cell line preferably is an immortalized kidney, bladder, liver, lung, cardiac muscle, smooth muscle, ovary or gastrointestinal cell. More preferably the immortalized human cell line is derived from an embryonic human kidney cell and most preferably it is cell line 293 T (ECACC: tsa201, ref. 96121229; DSM ACC2494)

The at least one transcription activator protein expressed by the immortalized cell line includes Simian virus T antigen, adenovirus E1A or E1B proteins, a protein encoded by the bovine papilloma virus early region DNA sequence and herpes virus IE proteins. Preferably the immortalized cell expresses at least two viral transcription activator proteins, e.g., a temperature sensitive SV40 T antigen and adenovirus E1A protein (such as the above cell line 293 T).

The promoter functionally linked to the DNA sequence coding for the human blood clotting factor preferably includes
(i) viral promoters being not stimulated by the activator protein expressed by the immortalized cell as defined above (such as SV40 and CMV);
(ii) housekeeping host promoters (albumin); and
(iii) tissue specific promoters (such as α-antitrypsin for liver). The most preferable promoter in accordance with the invention is a CMV promoter (while the transcription activator protein expressed by the immortalized cell is not stimulating said promoter).

In accordance with the invention the vector may carry additional viral promoters which are stimulated by said viral transcription activator proteins, but which are not functionally linked to the blood clotting factor. Such viral promoters are selected from promoters derived from adenovirus, rous sarcoma virus and cytomegalovirus. The vector may further comprise one or more of the following functional sequences: selection markers, regulatory sequences (e.g. PRE), etc.

The human blood clotting factor according to embodiment (1) of the invention includes, but is not limited to, factor IX, factor VIII, factor VII, factor V, von Willebrand factor (vWF) and the like.

In preferred embodiment (2) of the invention, the vector comprises a DNA sequence coding for factor VIII or a mutein thereof. Whereas recombinant

factor IX is in general structurally identical to the wild type protein isolated from blood plasma, several modified factor VIII expression constructs have been designed for recombinant expression. Considering the domain structure of the functional factor VIII polypeptide important interaction sites with vWF are located in the A3-domain (amino acid 1680-1689) and in the C2-domain (Kaufman & Pipe, *Haemophilia* (1998) 4, 370-379). Cleavage after 1689 was proposed to liberate factor VIII from vWF and permit factor VIII to interact with charged phospholipids. Recombinant factor VIII constructs lacking the vWF-binding site were shown to be extremely prone to proteolytic digestion when injected into factor VIII-deficient mice. Recombinant expression of truncated factor VIII constructs in mammalian cell cultures demonstrated that the complete deletion of the B-domain did not alter the biological activity of the corresponding factor VIII-like protein (Eaton et. al., *Biochemistry* (1986) 25, 8343-8347). In addition the observed expression rates of B-domain deleted constructs were significantly higher compared to wild-type factor VIII due to an increased mRNA-level in the cells (Pittman et al., *Blood* (1993) 81, 2925-2935). Four recombinant factor VIII products (Recombinate® Baxter HealthCare; Kogenate® and Kogenate FS® Bayer Corporation and Refacto® Wyeth, Genetics Institute) are currently on the market.

In preferred embodiment (3) of the invention the factor VIII mutein has at least one of the following mutations (a) to (d):

(a) Val at position 162 has been replaced by another neutral amino acid residue;

(b) Ser at position 2011 has been replaced by another hydrophilic amino acid residue;

(c) Val at position 2223 has been replaced by an acidic amino acid residue; and

(d) the B-domain between positions Arg740 and Glu1649 has been replaced by an Arg-rich linker peptide comprising 10 to 25, preferably 14 to 20 amino acid residues,

wherein said factor VIII numbering is relative to the amino acid sequence of wild-type factor VIII shown in SEQ ID NO: 2 (being the amino acid sequence of the mature peptide not including the 19 amino acid signal peptide, but including the entire B-domain (WO 99/29848)).

"Another neutral amino acid residue" in accordance with the present invention includes Gly, Ala, Leu, Ile, Met and Pro and preferably is Ala. The "another hydrophilic amino acid" includes Asn, Thr and Gln and preferably is Asn. The acidic amino acid residue is selected from Glu and Asp and preferably is Glu.

Among the factor VIII muteins of embodiment (3) it is preferred that the factor VIII mutein has at least one of the mutations (a), (b) and (c), more preferably at least one of the mutations (a) and (b), and most preferably all three mutations (a) to (c) as defined above. It is particularly preferred that the mutein comprises all three of the mutations V162A, S2011N and V2223E.

On the same token, the DNA sequence comprised by the vector of embodiment (4) of the invention has the mutations T485C, G6032A and T6668A relative to the DNA sequence of the mature wild-type factor VIII shown in SEQ ID NO: 1. In a preferred embodiment the DNA sequence also contains the quiet (i.e., silent) mutation T6816C (again said numbering being relative to the DNA sequence of the mature wild-type factor VIII).

Among the factor VIII muteins of embodiment (3) it is alternatively preferred that the factor VIII mutein has mutation (d) as defined above.

A preferred expression system of the invention utilizes a unique factor VIII mutein which - besides the point mutation (a) to (c) as defined herein before - partially or entirely lacks its B-domain, preferably a mutein where

the B-domain between position R740 and E1649 is replaced by a characteristic Arg-rich amino acid spacer as defined in (d) above. "Arg-rich" in accordance with the present invention means that said spacer comprises at least 3, preferably at least 4 Arg residues. In a most preferred embodiment said spacer consists of eight amino acids of the wilde type B-domain followed by eight amino acids of a variable domain (see Fig. 5A, SEQ ID NO: 9). In such construct having the B-domain modifications discussed herein before the proposed vWF-binding site remains unchanged to prevent an immediate proteolytic digestion of secreted factor VIII in the cell culture medium or later on in the blood of the treated patients. Only after specific activation by thrombin cleavage factor VIII will be released from vWF. The cDNA for the preferred factor VIII was constructed by assembling four DNA-fragments, e.g., as described in Example 1.

The protein of embodiment (3) of the invention may comprise additional N- or C-terminal sequences including, but not limited to, the natural export signal peptide (corresponding to amino acid residues -19 to -1 of the proteins shown in SEQ ID NOs 4, 13 and 15) or a fragment or analogue thereof, artificial peptides (e.g. oligo-His-tags for high-affinity purification) and the like.

The most preferred vector for the expression of factor VIII is vector pTGF8-1 shown in Fig. 2. The DNA sequence of said vector is shown in SEQ ID NO: 3, and it encompasses all five mutations addressed hereinbefore (the muteins T485C, G6032A, T6668A and T6816C (here: T1217C, G4088A, T4724A and T4872C) and a DNA sequence coding for the B-domain linker of SEQ ID NO: 9) and encodes the factor VIII mutein depicted in SEQ ID NO: 4.

Further most preferred vectors are pTGF8-2hyg-s and pTGF8-3, the common molecular structure of which is depicted in Fig. 6.

pTGF8-2hyg-s shown in SEQ ID NO: 12 contains the silent mutation T6816C only, resulting in a factor VIII mutein having the substitution of the B domain by the linker peptide SEQ ID NO. 9, but no further change in the primary protein structure referring to the wild type sequence SEQ ID NO. 2.

pTGF8-3 shown in SEQ ID NO: 14 contains mutations T485C, T6668A and T6816C, resulting in a factor VIII mutein showing amino acid substitutions V162A and V2223E referring to SEQ ID NO. 2 in addition to the substitution of the B domain as described above.

In the case of the production of factor VIII the culturing is performed in the presence of von Willebrand factor. The von Willebrand factor is preferably used in an amount of 10 to 100, more preferably 50 to 60 mol vWF per mol factor VIII (in the culture broth and/or in the factor VIII solution during the purification procedure (see below).

In preferred embodiment (4) of the present invention the human blood clotting factor is factor IX or a mutein thereof, preferably is wild-type factor IX shown in SEQ ID NO: 5. Suitable muteins of factor IX include point mutated and truncated forms of the factor IX. The most preferred vector for expression of factor IX are vectors pTGFG36 and pTG36hyg shown in Figs. 3 and 4, respectively.

In case of the production of factor IX, the culturing is preferably performed in the presence of vitamin K which may be present in an amount of 0.1 to 100 µg/ml culture broth, more preferably 1 to 20 µg/ml culture broth.

The method according to embodiment (1) of the invention further comprises the steps
(c) purifying the blood clotting factor isolated in step (b) and/or

(d) subjecting the blood clotting factor isolated in step (b) or purified in step (c) to a virus inactivation treatment.

Suitable purification steps include methods which were known in the art to maximize the yield of a pure, stable and highly active product and are selected from immunoaffinity chromatography, anion exchange chromatography, size exclusion chromatography, etc., and combinations thereof. In particular, detailed purification protocols for coagulation factors from human blood plasma are, e.g., disclosed in WO93/15105, EP0813597, WO96/40883 and WO 96/15140/50. They can easly be adapted to the specific requirements needed to isolate recombinant factors VIII and IX. For factor IX an effective protocol has been introduced containing an ammonium sulfate precipitation step followed by DEAE and HIC tentacle chromatography as well as heparin affinity chromatography (US5919909). Quantity and activity of the purified protein during and after the purification procedure may be monitored by ELISA and coagulation assays.

To overcome the problems of possible infectious contaminations in the purified protein samples or in the product directly obtained from the cell culture supernatant containing the secreted recombinant protein of choice, the samples and/or the culture supernatant might be treated with procedures for virus inactivation including heat treatment (dry or in liquid state, with or without tne addition of chemical substances including protease inhibitors). After virus inactivation a further purifying step for removing the chemical substances may be necessary. In particular, for factor VIII isolated from blood plasma the recovery of a high purity virus-inactivated protein by anion exchange chromatography was described (WO93/15105). In addition several processes for the production of high-purity, non-infectious coagulation factors from blood plasma or another biological sources have been reported. Lipid coated viruses are effectively inactivated by treating the potentially infectious material with a

hydrophobic phase forming a two-phase system, from which the water-insoluble part is subsequently removed. A further advantage has been proven to complement the hydrophobic phase treatment simultaneously or sequentially with a treatment with non-ionic biocompatible detergents and dialkyl or trialkyl phosphates. (WO 9636369, EP0131740, US 6,007,979). Non-lipid coated viruses require inactivation protocols consisting in treatment with non-ionic detergents follwed by a heating step (60-65 °C) for several hours (WO94/17834).

In view of the above results it is believed that the combination of an effective protein expression system based on a human cell line together with approved methods for inactivation of potentially dangerous infectious agents serve as a safe and easy to use-system for production of recombinant clotting factors.

Moreover, in accordance with embodiment (6) of the invention it is provided a superior factor VIII mutant. Said factor VIII mutant can be part of pharmaceutical compositions, can be used for preparing medicaments for treating hemophilia and can be applied in methods for treating hemophilia (embodiments (11) to (13) of the invention). The above pharmaceutical compositions and the above medicaments may comprise the factor VIII in a therapeutically effective dose, e.g., from 50 to 500 µg (with 200 ng factor VIII corresponding to one International Unit (IU)). Depending on the type of hemophilia, a patient receives an annual dose of factor VIII of up to 200,000 IU, which is usually administered in weekly or twice weekly doses.

The pharmaceutical compositions, medicaments or preparations applied in methods for treating hemophilia of embodiments (11) to (13) contains a therapeutically effective dose of the factor VIII mutein of embodiment (6) or the gene transfer vector of embodiment (9). In case of the former, it may further comprise pharmaceutically acceptable additives including

human serum albumin (HSA; preferably about 1 mg/ml solution); inorganic salts such as $CaCl_2$ (preferably 2 to 5 mM), amino acids such as glycine, lysine, and histidine (preferably 0.1 to 1 M per amino acid); disaccharides such as sucrose and/or trehalose (preferably 0.4 to 1 M); organic salts such as Na-citrate (preferably up to 50 mM); etc. The preparations may be aqueous or non-aqueous. In the latter case the major component is glycerol and/or polyethylene glycol (e.g., PEG-300). The preparation may also be in the dry form (to be dissolved in the desired solvent prior to administration).

As set forth above, the gene transfer vector in accordance with embodiment (9) of the invention can also be part of pharmaceutical compositions, can be used for preparing medicaments for treating hemophilia and can be applied in methods for treating hemophilia (embodiments (11) to (13) of the invention). Said pharmaceutical compositions and medicaments may further comprise suitable matrix formulations, e.g., lipids or hormones as discussed in WO 00/49147 (the disclosure thereof being herewith incorporated by reference). The pharmaceutical composition or medicament comprising the gene transfer vector or the gene transfer vector of the present invention may be administered orally, intravenously, intramuscularly, subcutaneously, tropically, through mucosa (including buccal, nasal spray) or by gene gun. Oral administration (e.g., in a micronized hormone dispersion) is preferred.

The factor VIII mutein of embodiment (6) of the invention is preferably as defined with reference to embodiment (3) above. Said FVIII mutein may further be prepared by standard recombinant techniques, e.g. a method comprising

(a) culturing a host cell transformed with the vector of embodiment (8) and/or comprising the DNA of embodiment (7) (which also includes culturing an immortalized human cell line stably expressing at least one

viral transcription activator protein and carrying a vector having a viral transcription promoter functionally linked to a DNA sequence coding for the human blood clotting factor, wherein said viral promoter is stimulated by said at least one viral transcription activator protein); and
(b) isolating the blood clotting factor from the culture broth. Suitable immortalized human cell lines, transcription activator proteins and viral promoters are those mentioned herein before. The immortalized human cell line utilized in said method preferably expresses two viral trascription activator proteins, most preferably temperature sensitive SV40 T antigen and adenovirus E1A protein. The method may further comprise the purification and virus inactivation steps (c) and (d) described herein before.

The commercially available cell line 293 T (ECACC: tsa201, ref. 96121229) was deposited with the DMSZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany) on February 20, 2001 under the depositary no. DSM ACC2494.

The invention is further illustrated by the following examples.

Examples

Example 1 - Cloning of factor VIII: The sequence for the recombinant factor VIII was obtained by reverse transcription from a complete human hepatocellular RNA pool. Afterwards four fragments (1/2, 3/4 , 5/6, 7/8) were amplified by standard PCR using primers designed to contain restriction sites. To fit together the fragments 3/4 and 5/6 the SmaI/SalI fragment from plasmid pBSFVIII3/4 was inserted blunt into the SalI site of pBSFVII5/6 to obtain pBSFVIII3/6. Next, the fragment 3/6 was obtained by digesting pBSFVIII3/6 with XhoI/BspHI and partially with Alw44I. This fragment and the PstI/Alw44I fragment from pBSFVIII1/2 were ligated in one step into the vector backbone of pBSFVIII1/2 digested with PstI and

XhoI by this means obtaining pBSFVIII1/6. The fragment 7/8 was obtained by digesting pBSFVIII7/8 with SmaI and partially with Mva1269I and ligated into pBSFVIII1/6 cut with XhoI and Mva1269I giving rise to pBSFVIII1/8. Finally the SmaI/XhoI fragment from pBSFIII1/8 was inserted blunt into the SalI site of Octagene Vector pTGFG67 (the production of said vector being disclosed in PCT/EP00/01368) resulting in the eukaryotic expression vector for the human factor VIII pTGF8-1 (s. figures 1 and 2). The resulting vector encodes a factor VIII mutein having the mutations V162A, S2011N and V2223E.

Example 2 - Cloning of factor IX: The vector pUC19 (MBI Fermentas) was digested with XbaI, treated with Klenow enzyme and religated. This XbaI deleted vector was then digested with EcoRI, treated with Klenow enzyme and religated in order to delete the EcoRI site. For insertion of an XbaI site into the SacI site of this vector it was digested with SacI, treated with T4-DNA-polymerase, dephosphorylated with alkaline phosphatase and ligated with the XbaI-linker CTCTAGAG (Biolabs #1032). Another XbaI-site was inserted by digesting the newly produced vector with HindIII, treating it with Klenow, dephosphorylating it with alkaline phosphatase and ligating it with the XbaI-linker CTCTAGAG (Biolabs #1032). This vector was named pUC19/X.

In order to destroy the XbaI-site present in the vector phGFP-S65T (Clontech) this vector was digested with XbaI, treated with Klenow enzyme and religated resulting in the vector pGFP/0. A 2.3 kb fragment containing the GFP-Gene was isolated after digesting pGFP/0 with MluI, treating it with Klenow enzyme and digesting it with BamHI. This fragment was inserted into the multiple cloning site of the vector pUC19/X which was digested with SalI, treated with Klenow enzyme and digested with BamHI. The resulting vector was named pTGFG1.

The oligonucleotides (Metabion) PRE-S (5'-GGG GTA CCA GCT TCG TAG CTA GAA CAT CAT GTT CTG GGA TAT CAG CTT CGT AGC TAG AAC ATC ATG TTC TGG TAC CCC-3'; SEQ ID NO: 10) and
PRE-AS (5'-GGG GTA CCA GAA CAT GAT GTT CTA GCT ACG AAG CTG ATA TCC CAG AAC ATG ATG TTC TAG CTA CGA AGC TGG TAC CCC-3'; SEQ ID NO: 11)
were hybridized and phosphorylated by kinase reaction, resulting in the insert PRE(ds).

The vector pTGFG1 was digested with EcoO109I, treated with Klenow enzyme and dephosphorylated with alkaline phosphatase. It was then ligated with the PRE(ds) insert, resulting in the vector pTGFG5.
The vector pUC19 (MBI Fermentas) was digested with SalI, treated with Klenow enzyme and dephosphorylated with alkaline phosphatase. It was ligated to the NotI-linker GCGGCCGC (Biolabs # 1045), resulting in the vector pUC19/N.

Factor IX cDNA was amplified from human liver cDNA (Clontech) using two primers overlapping the start and termination codon of the factor IX open reading frame resulting in a 1387 bp fragment containing the entire open reading frame. Restriction sites for EcoRI (upstream) and BamHI (downstream) were included at the end of each primer to facilitate cloning. Amplification was performed with Pwo DNA-polymerase (Boehringer Mannheim) in 50 µl reaction volume [10 mM Tris HCl pH 8.85, 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$] with 30 incubation cycles at 96°C for 1 min, 60°C for 1 min, 72°C for 2 min, followed by a final extension step at 72°C for 10 min.

Reaction products were ligated into the EcoRI- and BamHI-sites of pUC19 and transformed into *E. coli* DH5-α. Positive clones were selected. Sequences were confirmed by cycle sequencing (Amersham) from both

ends with labeled primers (IR-700) and automated analysis on the LiCor sequencing system (MWG, Biotech).

The following primers were used :
GGAATTCCGCAAAGGTTATGCAGCGCGTGAACATGATCATGGC (upstream; SEQ. ID NO: 16)
CGCGGATCCATTAAGTGAGCTTTGTTTTTTCCTTAATCC (downstream; SEQ. ID NO: 17)

A 1.4 kb fragment containing the open reading frame of the human clotting factor IX, isolated from a human cDNA library, was inserted into the PstI site of the above prepared vector pUC19/N which was digested with PstI, treated with T4-polymerase and dephosphorylated with alkaline phosphatase. From the resulting vector pUC19/N-FIX a 1.4 kb fragment containing the open reading frame of the human clotting factor IX was cut out by double-digestion with Hind III and NotI. This fragment was ligated to the 4.3 kb fragment of the HindIII and NotI double-digested vector pTGFG5 resulting in the vector pTGFG36 shown in Fig. 3. This vector is a preferred one for delivery of an expression cassette encoding factor IX into the cell, and its DNA sequence is provided in SEQ ID NO: 6.

Example 3 - Human cell line for protein expression: A preferred cell line is tsA201 (ECACC Ref.: 96121229) which is a transformed embryonal human kidney cell line (293, ECACC Number 85120602) stably expressing an SV40 temperature-sensitive T antigen (J. Membrane Biol. 1996;152:39; Gene 1995;156:235; PNAS USA 1994;91:12785; Pflügers Arch. 1994;427:136; J. Ger. Physiol. 1994;104:507; BioTechniques 1993;15:906). Other names for this cell line include 293tsA1609neo (Mol. Cell. Biol., 1987, 7:379) and 293T. This epithelial-like cell line has been used in a variety of functional expression assays and been reported to produce high levels of recombinant proteins. They can be cultivated in DMEM supplemented with 2mM glutamine and 10% FCS. For efficient pro-

duction of factor IX, the medium can be modified by addition of up to 100 µg/ml vitamin K (US4770999).

To simplify the purification of an expressed polypetide, cells can be cultivated in serum-free or protein-free medium containing suitable supplements. For stability reasons secreted factor VIII requires the presence of vWF in the medium (US5198349). Also the addition of lipoproteins, phospholipids, polyglycols, trace metals, heparin, non-ionic surfactants or cyclodextrin has been reported (EP0254076, US5679549, US5198349, US5250421, US5576194, EP0872487, WO94/11525, US5378612).

Example 4 - Calcium phosphate transfection of 293T cells for the transient production of factors VIII and IX: Confluent 293T cells were plated at low density in 10 cm dishes in 6 ml DMEM/10% FCS (10 µg/ml vitamin K for FIX) the day prior to transfection. Transfection was performed roughly according to Chen and Okayama (Mol. Cell Biol., 7:2745 (1987)). 12 µg of plasmid pTGF8-1 were transfected for the production of factor VIII and pTGFG36 for the production of factor IX. Six hours after transfection the medium was replaced by fresh one and the superantant was harvested three days post transfection and either further purified or analyzed without further purification by ELISA or coagulometry (see Examples 5 and 6).

Example 5 – Determination of FIX and FVIII concentration by ELISA:
Factor IX: Human recombinant factor IX levels in supernatant of transfected 293T cells were determined by ELISA using a goat polyclonal anti-human FIX (Enzyme Research Laboratories) as capture antibody. All incubations were performed for two hours in a humid chamber at 22° C. Plates (Dynex, Immulon-4) were coated with 100 µl of 8.8 µg antibody/ml coating buffer. Blocking is not required under the conditions described. Washing the plate four times (Encore 2000, Merck) with PBS-Tween® (0,1% v/v) is sufficient to block non-specific interactions.

After each further step washing was required to eliminate unbound proteins. 100 µl of supernatant treated with 10 µl 10mM PMSF and 10 µl 0.11 M sodium citrate were added to each well. Dilutions for samples and standard (human factor IX, house standard, Octapharma) were made in dilution buffer (HBS-BSA-EDTA-Tween®) and incubated at 100 µl/well. The detecting antibody was a peroxidase labelled goat polyclonal anti-FIX (Enzyme Research Laboratories) in a concentration of 1 µg antibody /ml dilution buffer and incubated at 100 µl/well. 150 µl ABTS (Roche) was added to each well as substrate, colorimetric reaction was detected at 405 nm after 1-2 hours. Results were calculated by linear regression of standard concentration versus standard absorbance and are summarized in the following table:

Number of cells [/ml]	Factor IX-concentration [ng/ml]	Clotting time [s]
$2,1 \times 10^5$	36	45
$8,7 \times 10^5$	20	79

Normal plasma: 37 – 39 s

Factor IX deficient plasma: 137 – 140 s

Factor VIII: Human recombinant factor VIII levels in culture filtrate of transfected 293T cells were determined by ELISA using an affinity purified polyclonal sheep anti FVIII:C preparation (F8C-EIA-C, Affinity Biologicals) as capture antibody. Coating was performed for two hours in a humid chamber at 22° C. Plates (Dynex, Immulon-4) were coated with 100 µl of a 100-fold antibody dilution in coating buffer (50 mM sodium carbonate pH 9.6). Washing the plate four times (Encore 2000, Merck) with PBS-Tween® (0,1% v/v) was sufficient to block non-specific interactions.

After each further step, washing was required to eliminate unbound proteins. 100 µl each of culture filtrate samples withdrawn from different

293T clones stably transfected with pTGF8-3 after 48h incubation were added to each well. Dilutions of FVIII standard (house standard, Octapharma) were made in dilution buffer (HBS-BSA-EDTA-Tween®) and incubated at 100 µl per well. For detection, a ready-to-use dilution of peroxidase labelled polyclonal anti-FVIII (F8C-EIA-D, Affinity Biologicals) was incubated for 60 min at 100 µl per well. For colorimetric reaction, a 5 mg O-Phenylenediamine (P-6912, Sigma) tablet was dissolved in 12 ml substrate buffer shortly before use and completed with 12 µl 30% H_2O_2. 150 µl of this substrate solution was added to each well and colorimetric recording was done in an MRX Reader (Dynex) at 490 nm after 10 min of incubation at room temperature in the dark and stopping of reaction by addition of 50 µl 2.5 M H_2SO_4 to each well. Results were calculated by linear regression of standard concentrations versus standard absorbances (Fig. 7 A) and are summarized in Fig. 7 B.

Example 6: Detection of Human Clotting Factor VIII and Factor IX Activity

The clotting activity of human recombinant factor VIII in supernatants of cell culture 293T cells (transfected by calcium phosphate precipitation with pTGF8-1 as described in Example 4) was determined as follows:

The clotting activity was assayed based on a partial thromboplastin time assay using Cephalin (phosphatidyl ethanolamine) activation with a manual coagulation instrument (ML-2, Instrumentation Laboratories). For the study, 100 µl undiluted supernatant from transfected 293 T-cells, 100 µl deficiency plasma (Progen) and 100 µl Cephalin (Instrumentation Laboratories) were incubated for 5 minutes at 37°C. Coagulation was started by adding 100 µl $CaCl_2$. Sample coagulation time was compared to normal plasma. The results are summarized in Fig. 5B. As can be seen from Fig. 5B, cell supernatant from cells transfected with pTGF8-1 shows a coagulation activity comparable to normal plasma while non transfected cells give a value equivalent to plasma lacking factor VIII.

A similar assay was performed with regard to factor IX. The results are shown in the Table of Example 5. For dependence of expression on the presence of vitamin K see Fig. 10.

Example 7 - Viral inactivation:

Viral inactivation was performed in accordance with the method of U.S. Patent No. 6,007,979. Namely, to a potentially infectious protein solution the following compounds were added subsequently, with stirring:

1. 0.2 ml of Tween® 80 and 0.06 ml of TNBP were added to 19.74 ml of the solution or

2. 0.2 ml of Triton® X-100 and 0.2 ml of TNBP were added to 19.6 ml of the solution.

1 ml of castor oil was added to preparations 1 and 2 which were then intensely extracted at room temperature for one hour.

Centrifugation was performed in each case for phase separation. For infectiousness control, samples of 1 ml each were repeatedly taken from the aqueous fraction.

Example 8 - Establishment of cell lines stably expressing factor VIII and factor IX: The preferred vectors pTGF8-1 and pTGFG36 comprise constructs for transient expression of factor VIII and factor IX , respectively, in mammalian cells. To enable a selection method for a stably transfected cell clone, a cassette for the hygromycin—B-phosphotransferase (HindIII-Mva 1260I fragment from TK-Hyg, Clontech) was subcloned into the SmaI site being present in both vectors. The resulting constructs (pTGF8-1-hyg and pTG36hyg) then comprise in *cis* the expression cassettes for the human factor VIII or factor IX with a CMV-promotor and a SV40-polyadenylation signal and a hygromycin-B-phosphotransferase expression cassette with the HSV thymidine kinase promoter and HSV thymidine kinase polyadenylation signal (see Fig. 4).

The vectors pTGF8-2hyg-s and pTGF8-3 (Fig. 6, SEQ ID NOs: 12 and 14) are derivatives of pTGF8-1hyg, in that point mutations V162A, S2011N and V2223E (pTGF8-2hyg-s) and S2011N (pTGF8-3) were reverted to wildtype sequence by a PCR- dependent method using the QuikChange® protocol (Stratagene).

The coding sequence for the clotting factors can be replaced by any other gene sequence of choice. These constructs allow for the establishment of stably expressing cell lines by calcium phosphate transfection and subsequent selection for hygromycine resistance. Additionally the plasmids contain a progesterone responsive element (PRE). In transient transfection experiments with pTG36hyg the production of about 40 ng active factor IX per ml culture medium could be shown by ELISA and coagulometric assay (see Examples 5 and 6).

For the production of factor IX 293T cells were cultivated in DMEM supplemented with 10% FCS and 10 µg/ml vitamin K (US Patent No. 4,770,999; see also Fig. 10). First the critical concentration of antibiotics for an effective selection of stably transfected 293T cells had to be established. For this purpose the cells were plated at low dilution and grown in the presence of 10 to 800 µg/ml hygromycin B. After two weeks at 200 µg/ml or higher nc cells were growing, so this concentration was chosen for the selection of stably transfected cells.

A typical transfection was performed in 10cm dishes with 293T cells split the day before at a ratio of 1:15. Using the calciumphosphate precipitation method (*Biotechniques* 1988 6:7 632-638) 12 µg plasmid per dish were transfected and two days later the medium was replaced with fresh one containing 200 µg/ml hygromycin B. After 2-3 weeks of selection the medium was tested by ELISA (see Example 5) for the presence of factor VIII or IX. Positive clones were isolated and transferred to a 24-well plate.

After screening by ELISA and activity determination positive clones were subjected to two further rounds of subcloning then expanded and aliquots of them frozen for further use and characterization.

Example 9: Proof of Phenotypic Uniformity of stably transfected cells by *in-situ* Immunofluorescent Detection of Factor VIII Expression:

Each, 5 x 10^7 293T cells stably transfected with pTGF8-3 (clone 49/19) and untransfected 293T cells (negative control) from adhesion cultures in DMEM + 9.1% FBS were detached from the culture dishes by trypsination, washed several times and resuspended in 5 ml PBS buffer.

2 µl of these cell suspensions were transferred to sterile microscopic glass slides and incubated at room temperature until all liquid was evaporated. Cells were fixed in 70% ethanol for 10 min and dried 5 min at room temperature. Slides were blocked against unspecific detection by incubation in a 10% dilution of FBS in PBS buffer. Primary antibody (sh antiFVIII:C F8C-EIA-C, Affinity Biologicals) was diluted 100-fold with PBS buffer containing 10% FBS and 0.1% saponine and incubated for 60 min at room temperature in a humid incubation chamber. After intense washing with PBS, a 100-fold dilution of the secondary antibody (rb anti sh CY3 conjugate 313-165-003, Jackson ImmunoResearch) was prepared and incubated in the way described above. Subsequently, the microscopic preparation was washed intensely and covered by a layer of 50% glycerol and a cover glass. Cells were visualized by white light- and by fluorescence microscopy (emission at 570 nm).
Results are depicted in Fig. 8.

Example 10: Thermal stability test on recombinant factor IX in culture filtrate:

Culture filtrate harvested from 293T cells 48 h after transient transfection with pTGFG36 in the presence of 100 µg/ml vitamin K and stored at -80 °C for 7 days was thawed quickly, distributed into 7 500 µl aliquots

which -then were subsequently submitted to the following thermal incubations:

sample	temp. (°C)	time (min)
1	0	240
2	20	30
3	20	60
4	20	240
5	37	30
6	37	60
7	37	240

Samples were chilled on ice and FIX activity was determined as outlined in Example 6 (double determinations). Results are shown in figure 9. Activity remained almost stable within incubation conditions up to 240 min 37 °C.

Claims

1. A factor VIII mutein in which the B-domain between positions Arg740 and Glu1649 has been replaced by an Arg-rich linker peptide having at least 3 Arg residues and comprising 10 to 25 amino acid residues, wherein said factor VIII numbering is relative to the mature wild-type factor VIII sequence shown in SEQ ID NO:2.

2. The factor VIII mutein of claim 1, wherein the factor VIII mutein has at least one of the following additional mutations (a), (b) and (c):
(a) Val at position 162 has been replaced by a neutral amino acid residue selected from Gly, Ala, Leu, Ile, Met and Pro;
(b) Ser at position 2011 has been replaced by a hydrophilic amino acid residue selected from Asn, Thr and Gln; and
(c) Val at position 2223 has been replaced by an acidic amino acid residue selected from Glu and Asp.

3. The factor VIII mutein of claim 2 which has at least one of the mutations (a) and (b).

4. The factor VIII mutein of claim 2, which has all three mutations (a), (b) and (c).

5. The factor VIII mutein according to any one of claims 2 to 4, wherein in mutation (a) Val at position 162 has been replaced by Ala, in mutation (b) Ser at position 2011 has been replaced by Asn, and/or in mutation (c) Val at position 2223 has been replaced by Glu.

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6. The factor VIII mutein according to any one of claims 1 to 5, wherein the Arg-rich linkerpeptide has 14 to 20 amino acid residues.

7. The factor VIII mutein according to any one of claims 1 to 6, wherein the linker comprises:

the amino acid sequence SFSQNSRH, and/or

the amino acid sequence QAYRYRRG.

8. The factor VIII mutein of claim 7, wherein the linker has the sequence SFSQNSRHQAYRYRRG.

9. The factor VIII mutein of claim 1, which comprises amino acids 1 to 1440 of SEQ ID NO: 4, 13 or 15.

10. A DNA sequence coding for the factor VIII mutein as defined in any one of claims 1 to 9.

11. The DNA sequence of claim 10, which has at least one of the mutations T485C, G6032A and T6668A relative to the DNA sequence of the mature wild-type factor VIII shown in SEQ ID NO: 1, preferably the DNA sequence comprises all three of said mutations.

12. A vector comprising the DNA as defined in claim 10 or 11.

13. The vector of claim 12 which is pTGF8-1, pTGT8-2hyg-s or pTGF8-3 as shown in SEQ ID NOs:3, 12 and 14, respectively.

14. The vector of claim 12 which is a gene transfer vector.

15. A host cell being transformed with a vector as defined in claim 12 and/or comprising a DNA sequence as defined in claim 10.

16. A method for the production of the factor VIII mutein as defined in any one of claims 1 to 9 which comprises
(a) culturing a transformed host cell as defined in claim 15; and
(b) isolating the factor VIII mutein from the culture broth.

17. A pharmaceutical composition comprising the factor VIII mutein as defined in any one of claims 1 to 9 or a gene transfer vector as defined in claim 14.

18. Use of the factor VIII mutein as defined in any one of claims 1 to 9 or a gene transfer vector as defined in claim 14 for preparing a medicament for treating hemophilia, preferably for treating hemophilia A.

19. The method of claim 16 which comprises
(a) culturing an immortalized human cell line stably expressing Simian virus T antigen and carrying a vector having a CMV promoter functionally linked to a DNA sequence coding for the factor VIII mutein.

20. The method of claim 19, wherein the immortalized human cell line is an immortalized kidney, bladder, liver, lung, cardiac muscle, smooth muscle, ovary or gastrointestinal cell.

21. The method of claim 20, wherein the immortalized human cell line is derived from an embryonic human kidney cell and preferably is cell line 293T (DSM ACC2494).

22. The method according to any one of claims 19 to 21, wherein the vector further comprises a selection marker and/or regulatory sequences.

23. The method according to any one of claims 19 to 22, wherein the culturing is performed in the presence of von Willebrand Factor (vWF).

24. The method according to any one of claims 16 and 19 to 23 which further comprises

(c) purifying the blood clotting factor isolated in step (b), and/or

(d) subjecting the blood clotting factor isolated in step (b) or purified in step (c) to a virus inactivation treatment.

25. An immortalized human cell line stably expressing at least one viral transcription activator protein and carrying a vector coding for a factor VIII mutein as defined in any one of the methods of claims 19 to 21.