Description

Title of Invention: BLOOD COAGULATION FACTOR VII AND VIIA DERIVATIVES, CONJUGATES AND COMPLEXES COMPRISING THE SAME, AND USE THEREOF

Technical Field

[1] The present invention relates to a blood coagulation factor VII derivative, a blood coagulation factor VIIa derivative, FacVII and FacVIIa conjugates each prepared by linking a polymer capable of extending the blood half-life to the derivative, FacVII and VIIa complexes each prepared by linking a carrier to the conjugate, genes encoding the FacVII and FacVIIa derivatives, expression vectors comprising the genes, transformants introduced with the expression vectors, a method for preparing the FacVII and FacVIIa derivatives using the transformants, a method for preparing the FacVIIa conjugate and complex, a FacVIIa complex prepared by the method, a pharmaceutical composition for preventing or treating hemophilia comprising the derivative, conjugate, or complex as an active ingredient, and a pharmaceutical composition for promoting blood coagulation comprising the derivative, conjugate, or complex as an active ingredient. Further, the present invention relates to a method for preventing or treating hemophilia or for promoting blood coagulation, comprising administering to a subject a therapeutically effective amount of the composition.

Background Art

[2] At present, there are an estimated 140 thousand people with hemophilia worldwide, showing an annual increase of 20%. Genetically, hemophilia occurs in one out of every ten thousand, but diagnosis or treatment is made only for approximately 25% of all patients. Based on etiology, hemophilia is largely divided into two types: one is hemophilia A that is caused by a lack of blood coagulation factor VII (Factor VII, FacVII) and accounts for 80% of the total hemophilia patients, and the other is hemophilia B that is caused by a lack of blood coagulation factor XI (Factor XI) and accounts for 20% of the total hemophilia patients. For the treatment of hemophilia, external administration of blood coagulation factors is given, but this treatment method is problematic in that 10-15% of all hemophilia A patients develop antibodies against the blood coagulation factor, and 1-3% of all hemophilia B patients develop antibodies against the blood coagulation factor.

[3] On the other hand, FacVII, which is a cause of hemophilia A accounting for more than a half of the hemophilia patients, is an enzyme that is mainly produced in the liver and composed of 406 amino acids, and includes gamma-carboxylation of glutamic acid at position 10, N-glycosylation of asparagines at positions 145 and 322, and O-

glycosylation of serines at positions 52 and 60. Further, FacVII has two EGF-like domains and one serine protease domain, and single-chain FacVII is activated through cleavage between arginine at position 152 and isoleucine at position 153 to generate two-chain FacVIIa consisting of a light chain and a heavy chain. Since activated FacVIIa acts through auxiliary blood clotting mechanism, unlike other blood co-agulation factors, antibodies are not produced even though injection of high-dose FacVIIa. Therefore, it can be used for the treatment of hemophilia A patients as well as patients having antibodies against FacVII due to the conventional therapies, and is known as a means of addressing the above described problems.

[4] However, antibodies against FacVIIa are not produced, but there is another problem of requiring high-dose, frequent administration because of a short blood half-life. Because of the short half-life, FacVIIa should be administered 2-3 times a day for the treatment of hemophilia, and this frequent administration also becomes a serious obstacle to the prevention of hemophilia. In order to solve the problem of short blood half-life, studies have suggested the known microencapsulation, liposome encap-sulation, and a variety of chemical modifications, but successful outcomes have not been reported yet. In particular, chemical modifications have been attempted such that the lysine residue or N-terminus on the surface of FacVIIa is chemically modified, or a carrier capable of extending blood half-life such as polyethylene glycol, albumin, transferrin, and immunoglobulin fragment is linked thereto, or a cysteine residue is inserted into a region not directly affecting the activity of FacVIIa to promote binding with other carrier. However, chemical modification of the lysine residue or N-terminus on the surface of FacVIIa reduces the ability of FacVIIa to bind with the membrane of platelet. When it is linked to other carrier, the carrier interferes with enzymatic ac-tivities. Insertion of cysteine residue induces formation of non-specific disulfide bond, consequently leading to a reduction in enzymatic activities. As such, many studies have been made to develop derivatives having an improved blood half-life without reducing the activity of FacVIIa, but no successful results have been reported yet.

[5] rVIIa-FP (CSL Behring) prepared by fusion of albumin to the C-terminus of FacVIIa is in the pre-clinical phase, and its blood half-life in rats was increased to 6.7 times higher than that of the native FacVIIa. However, it still has a very short half-life of 4.38 hrs, and thus is not suitable for the treatment and prevention of hemophilia. PEGLip-FVIIa (Omri) prepared by using a pegylated liposome formulation is also in the pre-clinical phase, but its blood half-life was only 2 times higher than that of the native FacVIIa.

[6] Two products, MAXY-VII (Bayer/Maxygen) prepared by Gla domain mutation and hyperglycosylation of FacVIIa to have a prolonged blood half-life and NN7128 (Novo/Neose) prepared by 40K PEG glycosylation to have a prolonged blood half-life

are under clinical studies, but their blood half-life was only 5 times higher than that of the native FacVIIa. Thus, they are not suitable for the effective treatment and prevention of hemophilia.

[7]

Disclosure of Invention

Technical Problem

[8] Based on this background, the present inventors have made many efforts to develop derivatives having improved blood half-life while retaining the maximum activities of FacVII and FacVIIa. As a result, they found that a derivative prepared by fusion of a part of the SOD1 (Superoxide Dismutase 1) sequence to the C-terminus of FacVII is easily able to bind with a carrier capable of extending the blood half-life such as polyethylene glycol, albumin, transferrin, and immunoglobulin fragment without reducing the activity of FacVII or FacVIIa, and in particular, an immunoglobulin Fc region, a non-peptidyl polymer, and a FacVII or FacVIIa derivative are site-specifically linked via a covalent bond to minimize the activity reduction and to remarkably increase the blood half-life of the conjugate, thereby completing the present invention.

Solution to Problem

[9] An object of the present invention is to provide a derivative of FacVII or its active form FacVIIa which has an amino acid sequence of blood coagulation factor VII (Factor VII, FacVII) or its active form, blood coagulation factor VIIa (Factor VIIa, FacVIIa) and a peptide linker at the C-terminus.

[10] Another object of the present invention is to provide a polynucleotide encoding the derivative of FacVII or its active form FacVIIa.

[11] Still another object of the present invention is to provide an expression vector comprising the polynucleotide.

[12] Still another object of the present invention is to provide a transformant introduced with the expression vector.

[13] Still another object of the present invention is to provide a method for preparing the derivative of FacVII or its active form FacVIIa using the transformant.

[14] Still another object of the present invention is to provide a conjugate of FacVII or its active form FacVIIa, which is prepared by linking a polymer capable of extending the blood half-life to the peptide linker of the derivative.

[15] Still another object of the present invention is to provide a complex of FacVII or its active form FacVIIa, which is prepared by linking a carrier capable of extending the blood half-life to one end of the conjugate.

[16] Still another object of the present invention is to provide a method for preparing the FacVIIa complex comprising the step of activating the FacVII complex.

[17] Still another object of the present invention is to provide a FacVIIa complex prepared by the above method.

[18] Still another object of the present invention is to provide a pharmaceutical composition for the prevention or treatment of hemophilia, comprising the derivative, conjugate, or complex as an active ingredient.

[19] Still another object of the present invention is to provide a pharmaceutical composition for blood coagulation, comprising the derivative, conjugate, or complex as an active ingredient.

[20] Still another object of the present invention is to provide a method for preventing or treating hemophilia, comprising the step of administering to a subject a therapeutically effective amount of the pharmaceutical composition for the prevention or treatment of hemophilia.

[21] Still another object of the present invention is to provide a method for promoting blood coagulation, comprising the step of administering to a subject a therapeutically effective amount of the pharmaceutical composition for blood coagulation.

[22]

Advantageous Effects of Invention

[23] The FacVII or FacVIIa derivative of the present invention is able to bind with a carrier capable of improving the blood half-life while maintaining the activity of FacVII or FacVIIa, and they can be widely used in the development of effective prophylactic or therapeutic agent for hemophilia.

[24]

Brief Description of Drawings

[25] FIG. 1a is a photograph showing the result of Western blot analysis of FacVII-ATKAVC expressed in 293F cell line;

[26] FIG. 1b is a photograph showing the result of Western blot analysis of a control group and FacVII-GGGGSC expressed in 293F cell line;

[27] FIG. 1c is a photograph showing the result of Western blot analysis showing the molecular weight difference of FacVII-ATKAVC and FacVII-SOD1 1-149 expressed in 293F cell line;

[28] FIG. 2 is a photograph showing the result of electrophoresis of the purified FacVII-ATKAVC;

[29] FIG. 3 is a photograph showing the result of electrophoresis of a FacVII-ATKAVC-PEG conjugate;

[30] FIG. 4a is a photograph showing the result of electrophoresis of a FacVIIa-ATKAVC-PEG-Fc conjugate;

[31] FIG. 4b is a photograph showing the result of Western blot analysis of the

FacVIIa-ATKAVC-PEG-Fc conjugate; and

[32] FIG. 5 is a graph of concentration-dependent absorbance showing in vitro activities of FacVII and FacVII-ATKAVC.

[33]

Best Mode for Carrying out the Invention

[34] In one aspect to achieve the above objects, the present invention provides a derivative of FacVII or its active form FacVIIa which has an amino acid sequence (SEQ ID NO. 4) of blood coagulation factor VII (Factor VII, FacVII) and a peptide linker at its C-terminus.

[35]

[36] As used herein, the term "blood coagulation factor VII (Factor VII, FacVII)" is, also called proconvertin, one of the factors involved in blood coagulation, and has a size of 48 kDa, and it is encoded by a gene having a size of 12.8 kb, and mainly produced in the liver, and one of vitamin K-dependent plasma proteins. It has been known that FacVII binds to blood coagulation factor III on the surface of extravascular tissues such as serine protease precursor and smooth muscle cells, tumor tissues, or activated leukocytes, and thus activates blood coagulation factors IX and X, leading to initiation of the extrinsic blood coagulation. In the present invention, FacVII may include a native FacVII, chemically modified FacVII derivatives that retain the normal activity of the native FacVII, and variants that have at least 80% amino acid sequence homology, preferably 85%, 90%, or 95% amino acid sequence homology, and more preferably 98% or 99% amino acid sequence homology with the native FacVII while they retain the normal activity of the native FacVII. However, the sequence homology is not limited thereto, as long as they exhibit the activity of the native FacVII.

[37] As used herein, the term "blood coagulation factor VIIa (Factor VIIa, FacVIIa)" means an active form of blood coagulation factor VII (Factor VII, FacVII), and single-chain FacVII is activated through cleavage between arginine at position 152 and isoleucine at position 153 to generate two-chain FacVIIa consisting of a light chain and a heavy chain. Since activated FacVIIa acts through auxiliary blood clotting mechanism, unlike other blood coagulation factors, antibodies are not produced even though injection of high-dose FacVIIa. In the present invention, FacVIIa may include a native FacVIIa, chemically modified FacVIIa derivatives that retain the normal activity of the native FacVIIa, and variants that have at least 80% amino acid sequence homology, preferably 85%, 90%, or 95% amino acid sequence homology, and more preferably 98% or 99% amino acid sequence homology with the native FacVII while they retain the normal activity of the native FacVIIa. However, the sequence homology is not limited thereto, as long as they exhibit the activity of the native FacVII.

[38] As used herein, the term "linker" basically refers to a means capable of linking two different fusion partners (e.g., biological polymers) using a hydrogen bond, an electrostatic interaction, a van der Waals force, a disulfide bond, a salt bridge, a hydrophobic interaction, a covalent bond or the like. Preferably, it may have at least one cysteine involved in at least one disulfide bond under physiological conditions or other standard peptide conditions (e.g., peptide purification conditions, peptide storage conditions). It is possible to use the cysteine as a reactive group linking the fusion partner as well as the disulfide bond. In addition, the linker functions to provide a predetermined space between carriers or functions as a hinge providing the fusion protein with flexibility or rigidity as well as it simply functions to link each fusion partner. In the present invention, the linker is, but not particularly limited to, a peptide linker that links the C-terminus of FacVII or FacVIIa to link a carrier capable of extending the blood half-life, and preferably a C-terminal cysteine residue of peptide linker. It may be preferably a partial sequence (SEQ ID NO. 30) of SOD1 (Superoxide dismutase 1), more preferably, a partial sequence (SEQ ID NO. 31) selected from 1 to 149 of SOD1 sequence, much more preferably from 1 to 90 of SOD1 sequence (SEQ ID NO. 32), even much more preferably from 1 to 25 of SOD1 sequence (SEQ ID NO. 33), and most preferably from 1 to 6 of SOD1 sequence (SEQ ID NO. 5).

[39] As used herein, the term "SOD1 (superoxide dismutase 1)" means an enzyme that catalyzes the disproportionation of the reactive oxygen, superoxide ion to oxygen and hydrogen peroxide, and is known to represent an important antioxidant defense in all cells exposed to oxygen. In the present invention, the SOD1 is used as a peptide linker capable of linking FacVII with the carrier capable of extending the blood half-life. SOD1 commonly found in the body is used as the linker, thereby reducing immunogenicity to the linker. VLKG (valine-leucine-lysine-glycine) within the peptide linker SOD1 sequence may be replaced by a self-cleavage site sequence IPRI (isoleucine-proline-arginine-isoleucine) that is recognized and cleaved by FacVIIa derivative. Owing to this replacement of the self-cleavage sequence, a linker region unnecessary for the activation can be removed by FacVIIa derivative upon activation.

[40] In the present invention, the self-cleavage site is a site containing a particular sequence, in which a polypeptide possesses the corresponding particular sequence in its own sequence and recognizes and cleaves it.

[41] As used herein, the term "FacVII derivative" means a modified FacVII that is composed of the amino acid sequence prepared by linking the peptide linker to the C-terminus of FacVII. The FacVII derivative of the present invention means the form prior to activation, and is changed to a FacVIIa derivative, when activated by a particular method. In the present invention, the FacVII derivative and FacVIIa derivative may have an equivalent meaning, except in a particular step, for example, a

preparation process of a conjugate or the like. In the present invention, the FacVII derivative is, but not particularly limited to, a polypeptide (SEQ ID NO. 9) prepared by linking ATKAVC (SEQ ID NO. 5) from 1 to 6 of the SOD1 sequence to the C-terminus of FacVII derivative, a polypeptide (SEQ ID NO. 13) prepared by linking GGGGSC (SEQ ID NO. 10) to the C-terminus of FacVII derivative, a polypeptide (SEQ ID NO. 14) prepared by linking the amino acid sequence from 1 to 149 of the SOD1 sequence to the C-terminus of FacVII derivative, a polypeptide (SEQ ID NO. 34) prepared by linking the amino acid sequence from 1 to 90 of the SOD1 sequence to the C-terminus of FacVII derivative, a polypeptide (SEQ ID NO. 35) prepared by linking the amino acid sequence from 1 to 25 of the SOD1 sequence to the C-terminus of FacVII derivative, a polypeptide (SEQ ID NO. 20) prepared by linking the amino acid sequence from 1 to 149 of the mutated SOD1 sequence to the C-terminus of FacVII derivative, a polypeptide (SEQ ID NO. 27) prepared by linking the amino acid sequence from 1 to 90 of the mutated SOD1 sequence to the C-terminus of FacVII derivative, or a polypeptide (SEQ ID NO. 24) prepared by linking the amino acid sequence from 1 to 25 of the mutated SOD1 sequence to the C-terminus of FacVII derivative.

[42] As used herein, the term "FacVIIa derivative" means an active form of the FacVII derivative, which has an amino acid sequence identical to that of the FacVII derivative, but is activated by cleavage between the amino acids at positions 152 and 153. In the present invention, the FacVIIa derivative is, but not particularly limited to, a polypeptide (SEQ ID NO. 9) prepared by linking ATKAVC (SEQ ID NO. 5) from 1 to 6 of the SOD1 sequence to the C-terminus of FacVIIa derivative, a polypeptide (SEQ ID NO. 13) prepared by linking GGGGSC (SEQ ID NO. 10) to the C-terminus of FacVIIa derivative, a polypeptide (SEQ ID NO. 14) prepared by linking the amino acid sequence from 1 to 149 of the SOD1 sequence to the C-terminus of FacVIIa derivative, a polypeptide (SEQ ID NO. 34) prepared by linking the amino acid sequence from 1 to 90 of the SOD1 sequence to the C-terminus of FacVII derivative , a polypeptide (SEQ ID NO. 35) prepared by linking the amino acid sequence from 1 to 25 of the SOD1 sequence to the C-terminus of FacVII derivative , a polypeptide (SEQ ID NO. 20) prepared by linking the amino acid sequence from 1 to 149 of the mutated SOD1 sequence to the C-terminus of FacVIIa derivative, a polypeptide (SEQ ID NO. 27) prepared by linking the amino acid sequence from 1 to 90 of the mutated SOD1 sequence to the C-terminus of FacVII derivative , or a polypeptide (SEQ ID NO. 24) prepared by linking the amino acid sequence from 1 to 25 of the mutated SOD1 sequence to the C-terminus of FacVIIa derivative.

[43]

[44] The present inventors investigated the characteristics for the activated FacVII, and

they intended to develop a derivative having the improved blood half-life without reducing the activity of FacVIIa. Non-activated FacVII is a single-chain FacVII by connecting light and heavy chains, and exposes only the N-terminus of light chain. However, when it becomes FacVIIa, the active site of heavy chain is exposed by cleavage between arginine at position 152 and isoleucine at position 153, and the exposed isoleucine at position 153 becomes the N-terminus of heavy chain. The N-terminus of heavy and light chains plays an important role in FacVIIa activation, and thus conjugation at the N-terminus may reduce the activity of FacVII, compared to the native FacVII.

[45] For this reason, the present inventors provide a FacVII derivative prepared by using a fragment of the SOD1 peptide sequence as a linker, the peptide fragment containing cysteine that is not exposed structurally to the outside and thus is not involved in the disulfide bond. In addition, a self-cleavage site sequence that can be recognized and cleaved by FacVIIa derivative is inserted in the peptide fragment linked as a linker, and thus a linker unnecessary for the activation can be removed. The present invention provides a FacVII derivative that has a fragment containing free cysteine of the SOD1 peptide at the C-terminus. It was found that a dimeric form of the FacVII derivative is produced at the lowest level during incubation, and the FacVII derivative is able to easily form a conjugate with a carrier capable of extending the blood half-life, thereby making up for the disadvantages of the native FacVII and the derivatives prepared by simple insertion of cysteine into FacVIIa.

[46] Therefore, a conjugate is prepared by linking to the C-terminus of the FacVII or FacVIIa derivative of the present invention a substance capable of remarkably improving the blood half-life, maintaining the blood coagulation function and remarkably increasing drug compliance, thereby preparing a product having more excellent effects of improving blood coagulation and preventing or treating hemophilia than the known products.

[47]

[48] In another aspect, the present invention provides a polynucleotide encoding the FacVII derivative, an expression vector comprising the polynucleotide, a transformant that is introduced with the expression vector to express the FacVII derivative, and a method for preparing the FacVII derivative using the transformant.

[49]

[50] The polynucleotide encoding the FacVII derivative provided in the present invention is, but not particularly limited to, a polynucleotide that is prepared by linking the FacVII-encoding region to the peptide linker-encoding region, and preferably a polynucleotide (SEQ ID NO. 8) encoding a polypeptide (SEQ ID NO. 9) that is prepared by linking ATKAVC (SEQ ID NO. 5) from 1 to 6 of the SOD1 sequence to

the C-terminus of FacVII derivative, a polynucleotide (SEQ ID NO. 12) encoding a polypeptide (SEQ ID NO. 13) that is prepared by linking GGGGSC (SEQ ID NO. 10) to the C-terminus of FacVII derivative, a polynucleotide (SEQ ID NO. 15) encoding a polypeptide (SEQ ID NO. 14) that is prepared by linking 1 to 149 amino acids of the SOD1 sequence to the C-terminus of FacVII derivative, a polynucleotide (SEQ ID NO. 21) encoding a polypeptide (SEQ ID NO. 20) that is prepared by linking 1 to 149 amino acids of the mutated SOD1 sequence to the C-terminus of FacVII derivative, a polynucleotide (SEQ ID NO. 28) encoding a polypeptide (SEQ ID NO. 27) that is prepared by linking 1 to 90 amino acids of the mutated SOD1 sequence to the C-terminus of FacVII derivative, or a polynucleotide (SEQ ID NO. 25) encoding a polypeptide (SEQ ID NO. 24) that is prepared by linking 1 to 25 amino acids of the mutated SOD1 sequence to the C-terminus of FacVII derivative.

[51]

[52] The expression vector comprising the polynucleotide encoding the FacVII derivative provided in the present invention is, but not particularly limited to, a vector capable of replicating and/or expressing the polynucleotide in eukaryotic or prokaryotic cells, including mammalian cells (e.g., human, monkey, rabbit, rat, hamster, mouse cells, etc.), plant cells, yeast cells, insect cells or bacterial cells (e.g., E. coli, etc.), and preferably a vector that is operably linked to a proper promoter to express the polynucleotide in a host cell and contains at least one selection marker. More preferably, it may be an expression vector prepared by introduction of the polynucleotide into a phage, a plasmid, a cosmid, a mini-chromosome, a viral vector, or a retroviral vector. Most preferably, it may be an expression vector pX0GC-FVII-ATKAVC including the FacVII derivative-encoding polynucleotide that is prepared by linking the polynucleotide encoding ATKAVC (SEQ ID NO. 5) from 1 to 6 of the SOD1 sequence to the 3'-terminus of FacVII gene, an expression vector pX0GC-FVII-GGGGSC including the FacVII derivative-encoding polynucleotide that is prepared by linking the polynucleotide encoding GGGGSC (SEQ ID NO. 10) to the 3'-terminus of FacVII gene, an expression vector pX0GC-FVII-SOD1 1-149 including the FacVII derivative-encoding polynucleotide that is prepared by linking the polynucleotide encoding the amino acid sequence (SEQ ID NO. 14) from 1 to 149 of the SOD1 sequence to the 3'-terminus of FacVII gene, an expression vector pX0GC-FVII-SOD1 IPRI including the FacVII derivative-encoding polynucleotide (SEQ ID NO. 21) that is prepared by linking the polynucleotide encoding 1 to 149 amino acids of the mutated SOD1 sequence to the 3'-terminus of FacVII gene, an expression vector pX0GC-FVII-SOD1 1-90 IPRI including the FacVII derivative-encoding polynucleotide (SEQ ID NO. 28) that is prepared by linking the polynucleotide encoding 1 to 90 amino acids of the mutated SOD1 sequence to the 3'-terminus of FacVII gene, or an expression vector

pX0GC-FVII-SOD1 1-25 IPRI including the FacVII derivative-encoding polynucleotide (SEQ ID NO. 25) that is prepared by linking the polynucleotide encoding 1 to 25 amino acids of the mutated SOD1 sequence to the 3'-terminus of FacVII gene.

[53]

[54] The transformant introduced with the expression vector provided in the present invention is, but not particularly limited to, bacterial cells such as E. coli, Streptomyces, and Salmonella typhimurium; yeast cells such as Pichia pastoris; insect cells such as Drosophila and Spodoptera Sf9 cells; animal cells such as CHO, COS, NSO, 293, and Bowes melanoma cells; or plant cells, which are transformed by introduction of the expression vector. It may be preferably a transformant prepared by introduction of the expression vector into 293F or CHO cell line, and most preferably HMF709 prepared by introduction of the expression vector pX0GC-FVII-ATKAVC into CHO cell line.

[55]

[56] The method for preparing the FacVII derivative provided in the present invention comprises the steps of (i) culturing the transformant so as to obtain a culture solution; and (ii) recovering the FacVII derivative from the culture solution.

[57] The method further comprises the step of activating the recovered FacVII derivative, thereby preparing the FacVIIa derivative from the prepared FacVII derivative. The activation method is the same as described above.

[58]

[59] The present inventors prepared an expression vector pX0GC-FVII-ATKAVC including the FacVII derivative-encoding polynucleotide that is prepared by linking the polynucleotide encoding ATKAVC (SEQ ID NO. 5) from 1 to 6 of the SOD1 sequence to the 3'-terminus of FacVII gene (Example 2-1), and the expression vector was introduced into 293F cell line (Example 3-1) or CHO cell line (Example 3-2) so as to obtain a transformant. Subsequently, the FacVII derivative was expressed from the transformant, and the expressed FacVII derivative was purified (Example 4, FIG. 2). The expressed FacVII derivative was activated to prepare the FacVIIa derivative, followed by comparison of its activity with that of native FacVIIa (Example 6 and FIG. 4). As a result, the FacVIIa derivative prepared from the FacVII derivative of the present invention was found to show the activity equivalent to that of native FacVIIa. Thus, a clone showing the highest expression level of FacVII derivative was selected from the transformants prepared by introduction of the expression vector pX0GC-FVII-ATKAVC into CHO cells, and was designated as "HMF709", and deposited at the Korean Collection for Type Culture, Korea Research Institute of Bioscience and Biotechnology (111 Gwahangno, Yuseong-gu, Daejeon, Korea) under accession number "KCTC12022BP".

[60]

[61] In still another aspect, the present invention provides a conjugate of FacVII or its active form FacVIIa which is prepared by linking a polymer capable of extending the blood half-life to the peptide linker of the FacVII derivative.

[62] The polymer of the present invention may be a polymer such as polyethylene glycol capable of extending the blood half-life, and selected from protein carriers such as immunoglobulin fragment, transferrin, antibody, and albumin.

[63] The present invention provides a conjugate that is prepared by linking the FacVII derivative with the protein carrier using a non-peptidyl polymer as a linker in vitro without using a genetic recombination method.

[64] The non-peptidyl polymer of the present invention refers to a non-peptidyl polymer designed to resist to the degradation by various exzymes or immune molecules in the blood or serum. The non-peptidyl polymer which is not limited by the followed, may be selected from the group consisting of polyethylene glycol, polypropylene glycol, ethylene glycol-propylene glycol copolymers, polyoxyethylated polyols, polyvinyl alcohols, polysaccharides, dextrans, polyvinyl ethyl ethers, biodegradable polymers, lipid polymers, chitins, hyaluronic acids, and a combination thereof. And the non-peptidyl polymer can be linked to each other via any kind of covalent bond except peptide bond. Further, the derivatives thereof known in the art and derivatives easily prepared by any known technique in the art are also within the scope of the present invention. In the present invention, the non-peptidyl polymer may be linked to the peptide linker of the FacVII derivative or the FacVIIa derivative. The non-peptidyl polymer may be linked to the various binding sites of the peptide linker. Preferably, the non-peptidyl polymer may be linked to the C-terminus of peptide linker present at the FacVII derivative or the FacVIIa derivative.

[65] The non-peptidyl polymer can comprise reactive group which may include, but is not limited to, a aldehyde, a propionaldehyde, a butyraldehyde, a maleimide or a succinimide(succinimidyl propionate, succinimidyl carboxymethyl, hydroxy succinimidyl, or succinimidyl carbonate). In addition, the non-peptidyl polymer may have a single reactive group or double reactive groups. If the non-peptidyl polymer comprises two or more reactive groups, it can be linked to the liker of FacVII derivative at one reactive group, and also linked to another carrier such as the antibodies, the immunoglobulin fragments, albumin, or transferrin at other reactive group. For example, when the non-peptidyl polymer has a reactive aldehyde group at one end and a maleimide, ortho pyridyl disulfide or thiol reactive group at the other end, non-specific reaction can be minimized, and it is effective in the selective binding of the FacVII derivative or the FacVIIa derivative and carrier at both ends of the non-peptidyl polymer. A final product produced by reductive alkylation due to the aldehyde bond may be more stable

than an amide bond. In addition, the aldehyde reactive group selectively reacts with the amino terminus of the carrier at a low pH, and may form a covalent bond with a lysine residue at a high pH, for example, pH 9.0.

[66] In still another aspect, the present invention provides a complex of FacVII or its active form FacVIIa which is prepared by linking the derivative of FacVII or its active form FacVIIa with an immunoglobulin Fc region via the non-peptidyl polymer.

[67] The FacVII complex that is linked to a carrier such as antibody, immunoglobulin fragment, albumin, and transferrin, in particular, the immunoglobulin Fc via the non-peptidyl polymer may be prepared by the steps of (1) covalently linking a non-peptidyl polymer having an aldehyde or succinimide derivative reactive group at its one end to the amine group of immunoglobulin Fc; (2) recovering a conjugate that comprises the immunoglobulin Fc region covalently linked with the non-peptidyl polymer at the amine group, from the reaction mixture of step (1); (3) covalently linking the FacVII derivative to the other end of the non-peptidyl polymer having a maleimide, ortho pyridyl disulfide, or thiol reactive group in the recovered conjugate so as to produce a FacVII complex having the immunoglobulin Fc region and the FacVII derivative at each end of the non-peptidyl polymer; and (4) activating the FacVII conjugate produced in step (3) so as to produce a FacVIIa complex having FacVIIa and the immunoglobulin Fc region linked via the non-peptidyl polymer.

[68] Further, the FacVII complex may be prepared by the steps of (1) covalently linking a non-peptidyl polymer having a maleimide, ortho pyridyl disulfide, or thiol reactive group at its one end to the C-terminal thiol group of FacVII derivative; (2) recovering a conjugate that includes the FacVII derivative covalently linked with the non-peptidyl polymer, from the reaction mixture of step (1); (3) covalently linking the immunoglobulin Fc region to the other end of the non-peptidyl polymer having an aldehyde or succinimide derivative reactive group in the recovered conjugate so as to produce a FacVII complex having the immunoglobulin Fc region and the FacVII derivative at each end of the non-peptidyl polymer; and (4) activating the FacVII conjugate produced in step (3) so as to produce a FacVIIa complex having FacVIIa and the immunoglobulin Fc region linked via the non-peptidyl polymer.

[69] Further, the FacVIIa complex may be prepared by the steps of (1) covalently linking a non-peptidyl polymer having a maleimide, ortho pyridyl disulfide, or thiol reactive group at its one end to the C-terminal thiol group of FacVIIa derivative; (2) recovering a conjugate that includes the FacVII derivative covalently linked with the non-peptidyl polymer, from the reaction mixture of step (1); and (3) covalently linking the immunoglobulin Fc region to the other end of the non-peptidyl polymer having an aldehyde or succinimide derivative reactive group in the recovered conjugate so as to produce a FacVIIa complex having the immunoglobulin Fc region and the FacVIIa

derivative at each end of the non-peptidyl polymer.

[70] On the other hand, the non-peptidyl polymer may include two or three reactive ends, and the two or three reactive ends may be the same as or different from each other. For example, it may have a maleimide group at one end and an aldehyde group, a propionaldehyde group, or a butyraldehyde group at the other end. When poly(ethylene glycol) having hydroxy reactive groups at both ends thereof is used as the non-peptidyl polymer, the hydroxy group may be activated to various reactive groups by known chemical reactions, or a poly(ethylene glycol) having a commercially available modified reactive group may be used so as to prepare the FacVII conjugate and complex of the present invention.

[71] Therefore, the non-peptidyl polymer included in the FacVII conjugate and complex of the present invention may be preferably a non-peptidyl polymer having a methyl group at one end and a maleimide, ortho pyridyl disulfide or thiol reactive group at the other end, and more preferably a non-peptidyl polymer having a maleimide, ortho pyridyl disulfide or thiol reactive group at one end and an aldehyde or succinimide derivative reactive group at the other end, and most preferably a non-peptidyl polymer having a maleimide reactive group and an aldehyde reactive group at both ends, respectively.

[72] The FacVII derivative that is used in the preparation of the conjugate or complex using the FacVII derivative of the present invention may be an inactive form or an activated FacVIIa derivative. However, the use of FacVII is preferred in order to prevent degradation due to the activated FacVIIa during the conjugate preparation using the FacVIIa derivative.

[73] As the carrier, the Fc regions may be obtained from native forms isolated from humans and other animals including cows, goats, pigs, mice, rabbits, hamsters, rats and guinea pigs. In addition, the immunoglobulin Fc region may be an Fc region that is derived from IgG, IgA, IgD, IgE and IgM, or that is made by combinations thereof or hybrids thereof. Preferably, it is derived from IgG or IgM, which are among the most abundant proteins in human blood, and most preferably from IgG, which is known to enhance the half-lives of ligand-binding proteins. Immunoglobulin Fc may be obtained from a native immunoglobulin by isolating whole immunoglobulins from human or animal organisms and treating them with a specific proteolytic enzyme, and also may be obtained from transformed cells by recombination technique. Preferably, it is a recombinant human immunoglobulin Fc region from E.coli. On the other hand, IgG is divided into IgG1, IgG2, IgG3 and IgG4 subclasses, and the present invention includes combinations and hybrids thereof. Preferred are the IgG2 and IgG4 subclasses, and most preferred is the Fc region of IgG4 rarely having effector functions such as CDC (complement dependent cytotoxicity).

[74] That is, as the drug carrier of the present invention, the most preferable immunoglobulin Fc region is a human IgG4-derived non-glycosylated Fc region. The human-derived Fc region is more preferable than a non-human derived Fc region, which may act as an antigen in the human body and cause undesirable immune responses such as the production of a new antibody against the antigen.

[75] The peptide linker which is used in the fusion protein obtained by a conventional inframe fusion method has drawbacks in that it is easily *in-vivo* cleaved by a proteolytic enzyme, and thus a sufficient effect of increasing the serum half-life of the active drug by a carrier cannot be obtained as expected. However, in the present invention, the polymer having resistance to the proteolytic enzyme can be used to maintain the serum half-life of the peptide being similar to that of the carrier. Therefore, any non-peptidyl polymer can be used without limitation, as long as it is a polymer having the aforementioned function, that is, a polymer having resistance to the *in-vivo* proteolytic enzyme. The non-peptidyl polymer has a molecular weight in the range of 1 to 100 kDa, and preferably of 1 to 40 kDa. The non-peptidyl polymer of the present invention, linked to the immunoglobulin Fc region, may be one polymer or a combination of different types of polymers.

[76]

[77]

[78] In one embodiment of the present invention, in vitro activity of the FacVII conjugate was determined. The present invention is intended to minimize a reduction in the activity by site-specific conjugation of FacVII and the non-peptidyl polymer. Thus, the activities of FacVII-ATKAVC and FacVII-ATKAVC-40kDa PEG were determined using the native FacVII and FacVII-40 kDa PEG as a control group (Example7). As a result, it was found that in vitro activity of the N-terminal PEGylated FacVII-40 kDa PEG was approximately 11%, compared to that of FacVII, and in vitro activity of the C-terminal PEGylated FacVII-ATKAVC-40 kDa PEG was approximately 29%, compared to that of FacVII-ATKAVC. That is, the C-terminal PEGylated FacVII-ATKAVC-40 kDa PEG maintains an activity approximately 2.5 times higher than EC_{50} of the N-terminal PEGylated FacVII-40 kDa PEG, indicating that the FacVII activity can be maintained at a higher level by site-specific conjugation using ATKAVC (Table 2).

[79]

[80] In another embodiment, in vitro activity of the complex prepared by linking the non-peptidyl polymer and the immunoglobulin Fc region to the FacVII conjugate was determined (Example 8). As a result, it was found that in vitro activity of FacVIIa-ATKAVC-PEG-Fc was approximately 45%, compared to that of FacVIIa-ATKAVC (Table 3), indicating that the complex linked with the non-peptidyl

polymer and the immunoglobulin Fc region has the carrier capable of improving the blood half-life while maintaining the FacVII activity, thereby being widely used in the development of more effective prophylactic or therapeutic agent for hemophilia.

[81]

[82] In still another aspect, the present invention provides a method for preparing a FacVIIa conjugate comprising the step of activating the FacVII conjugate, and a FacVIIa conjugate prepared by the method. In detail, the method for preparing the FacVIIa conjugate may comprise the steps of (i) covalently linking a non-peptidyl polymer capable of extending the blood half-life to the C-terminal thiol group of the FacVII derivative; (ii) recovering the FacVII conjugate that is composed of the non-peptidyl polymer linked to the FacVII derivative; and (iii) activating the recovered FacVII conjugate so as to produce a FacVIIa conjugate having the non-peptidyl polymer linked to the FacVIIa region.

[83] In addition, the method comprises the steps of (i) covalently linking the non-peptidyl polymer capable of extending the blood half-life to the C-terminal thiol group of the FacVIIa derivative; and (ii) recovering the FacVIIa conjugate that is composed of the non-peptidyl polymer linked to the FacVIIa derivative so as to produce a FacVIIa conjugate having the non-peptidyl polymer linked to the FacVIIa region.

[84]

[85] The non-peptidyl polymer capable of extending the blood half-life used in the method is the same as described above, and the method for activating FacVII or FacVII conjugate is, but not particularly limited to, an on-column activation (auto-activation) of activating the FacVII or FacVII conjugate by attaching it to an anion exchange column or an in-solution activation of activating the FacVII or FacVII conjugate by reacting it in a solution phase. In particular, the on-column activation is also called solid-phase activation, and is performed by "auto-activation" after attachment of the FacVII or FacVII conjugate to the anion exchange column without additional components. In contrast, the in-solution activation is a method of inducing FacVII activation, considering various factors needed in FacVII activation, for example, calcium ion concentration, pH, temperature, and FacVII concentration.

[86] The present inventors demonstrated that the blood half-life of the conjugate prepared by linking the immunoglobulin fragment to the native FacVII via the non-peptidiyl linker was increased to approximately 200 times, compared to the native FacVII having no immunoglobulin fragment (Korean Patent Application No. 2010-0062860). It is well known that the increased half-life is not attributed to FacVII, but attributed to the non-peptidiyl linker and the immunoglobulin fragment. Therefore, the conjugate prepared by using the prepared FacVII derivative is also expected to have the increased half-life.

[87]

[88] In still another aspect, the present invention provides a pharmaceutical composition for the prevention or treatment of hemophilia or a pharmaceutical composition for blood coagulation, comprising the derivative of FacVII or its active form FacVIIa, the conjugate of FacVII or its active form FacVIIa, the complex of FacVII or its active form FacVIIa, as an active ingredient.

[89] In addition, the present invention provides a method for preventing or treating hemophilia or for promoting blood coagulation, comprising administering to a subject a therapeutically effective amount of the pharmaceutical composition.

[90]

[91] As used herein, the term "prevention" means all of the actions by which the occurrence of hemophilia is restrained or retarded by concurrent administration of the composition of the present invention, and the term "treatment" means all of the actions by which the symptoms of diabetes have taken a turn for the better or been modified favorably by concurrent administration of the composition of the present invention.

[92] In the present invention, the method for promoting blood coagulation is to promote the action of blood coagulation factor by preparation of derivatives, conjugates, or complexes having remarkably increased blood half-life from blood coagulation factor FacVII or its active form FacVIIa having a short half-life.

[93] Further, the pharmaceutical composition of the present invention may include a pharmaceutically acceptable carrier. As used herein, the term "pharmaceutically acceptable carrier" refers to a carrier or diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. For oral administration, the pharmaceutically acceptable carrier may include a binder, a lubricant, a disintegrant, an excipient, a solubilizer, a dispersing agent, a stabilizer, a suspending agent, a coloring agent, and a flavor. For injectable preparations, the pharmaceutically acceptable carrier may include a buffering agent, a preserving agent, an analgesic, a solubilizer, an isotonic agent, and a stabilizer. For preparations for topical administration, the pharmaceutically acceptable carrier may include a base, an excipient, a lubricant, and a preserving agent. . The pharmaceutical composition of the present invention may be formulated into a variety of dosage forms in combination with the aforementioned pharmaceutically acceptable carriers. For example, for oral administration, the pharmaceutical composition may be formulated into tablets, troches, capsules, elixirs, suspensions, syrups or wafers. For injectable preparations, the pharmaceutical composition may be formulated into a unit dosage form, such as a multi-dose container or an ampule as a single-dose dosage form. The pharmaceutical composition may be also formulated into solutions, suspensions, tablets, pills, capsules and long-acting preparations.

[94] On the other hand, examples of the carrier, the excipient, and the diluent suitable for the pharmaceutical formulations include lactose, dextrose, sucrose, sorbitol, mannitol, xylitol, erythritol, maltitol, starch, acacia rubber, alginate, gelatin, calcium phosphate, calcium silicate, cellulose, methylcellulose, microcrystalline cellulose, polyvinylpyrrolidone, water, methylhydroxybenzoate, propylhydroxybenzoate, talc, magnesium stearate and mineral oils. In addition, the pharmaceutical formulations may further include fillers, anti-coagulating agents, lubricants, humectants, flavors, and antiseptics.

[95]

[96] In still another aspect, the present invention provides a method for treating hemophilia, comprising administering to a subject having hemophilia a therapeutically effective amount of the pharmaceutical composition for the prevention or treatment of hemophilia including the derivative, conjugate, or complex as an active ingredient. In this regard, the pharmaceutical composition may be administered alone or in combinations with other therapeutic agents simultaneously or sequentially.

[97]

[98] As used herein, the term administration means introduction of a predetermined amount of a substance into a patient by a certain suitable method. The composition may be administered via any of the common routes, as long as it is able to reach a desired tissue. A variety of modes of administration are contemplated, including intraperitoneal, intravenous, intramuscular, subcutaneous, intradermal, oral, topical, intranasal, intrapulmonary and intrarectal, but the present invention is not limited to these exemplified modes of administration. However, since peptides are digested upon oral administration, active ingredients of a composition for oral administration should be coated or formulated for protection against degradation in the stomach. Preferably, the multimer may be administered in an injectable form. In addition, the pharmaceutical composition may be administered using a certain apparatus capable of transporting the active ingredients into a target cell.

[99] Further, the pharmaceutical composition of the present invention can be determined by several related factors including the types of diseases to be treated, administration routes, the patient's age, gender, weight and severity of the illness, as well as by the types of the drug used as an active component.

[100] The pharmaceutical composition of the present invention shows excellent in-vivo duration of efficacy and titer, thereby remarkably reducing the number and frequency of administration thereof for preventing or treating hemophilia or for promoting blood coagulation.

[101]

Mode for the Invention

[102] Hereinafter, the present invention will be described in more detail with reference to Examples. However, these Examples are for illustrative purposes only, and the invention is not intended to be limited by these Examples.

[103]

[104] **Example 1: Preparation of FacVII gene-containing expression vector**

[105] First, human Factor VII gene containing a signal sequence was obtained using a Polymerase Chain Reaction(PCR) technique. For amplification of Factor VII (FacVII) gene, a human fetal liver cDNA library (TAKARA BIO USA) was used as a template, and forward and reverse primers of the following SEQ ID NOs. 1 and 2 were used to perform PCR (95°C 1 minute denaturation; 30 cycles (95°C 30 seconds, 60°C 30 seconds and 68°C 90 seconds); 68°C 5 minutes). At this time, for easy cloning, the recognition site for the restriction enzyme BamHI was inserted into the primer of SEQ ID NO. 1 and the recognition site for the restriction enzyme XhoI was inserted into the primer of SEQ ID NO. 2. Subsequently, a nucleotide sequence (SEQ ID NOs. 3 and 4) of the PCR product of approximately 1.3 kb obtained by PCR was examined.

[106]

[107] VIIBHISS F: 5'-cccggatccatggtctcccaggccctcaggctcc-3' (SEQ ID NO. 1)

[108] VIIXhoIAS R: 5'-gggctcgagctagggaaatggggctcgcagg-3' (SEQ ID NO. 2)

[109]

[110] In order to express the obtained PCR product under the control of CMV promoter, it was cloned into an animal cell expression vector pX0GC. The pX0GC vector is an expression vector including one or more CCGCCC repeat sequence-removed DHFR promoter and a DHFR-encoding nucleotide sequence operably linked thereto (Korean Patent No. 880509). Specifically, the PCR product was digested with the restriction enzymes, BamHI and XhoI at 37°C for 2 hours, and applied to a PCR purification kit (Qiagen, USA) so as to obtain the cleaved DNA fragment. The DNA fragment was mixed with the pX0GC vector treated with the restriction enzymes BamHI and XhoI, and cloned using T4 DNA ligase, thereby preparing an expression vector including FacVII gene.

[111]

[112] **Example 2: Preparation of expression vector for expression of various recombinant FacVII derivatives**

[113] The FacVII gene-containing expression vector (pX0GC-FVII) prepared in Example 1 was used to obtain a polynucleotide encoding a FacVII derivative which has a partial sequence of SOD1 (Superoxide Dismutase 1, SEQ ID NO. 30) at the C-terminus of FacVII, and an expression vector capable of expressing the derivative was prepared.

[114]

[115] **Example 2-1: Preparation of recombinant FacVII derivative-expressing vector, pX0GC-FVII-ATKAVC**

[116] A recombinant FacVII derivative-expressing vector pX0GC-FVII-ATKAVC, which contains a polynucleotide further having a polynucleotide encoding the sequence from 1 to 6 of the SOD1 sequence at the 3'-terminus of FacVII gene included in the expression vector pX0GC-FVII prepared in Example 1, was prepared. In detail, the expression vector pX0GC-FVII was used as a template, and forward and reverse primers of the following SEQ ID NOs. 6 and 7 were used to perform PCR (95°C 1 minute denaturation; 30 cycles (95°C 60 seconds, 60°C 60 seconds and 68°C 90 seconds); 68°C 5 minutes). At this time, for easy cloning, the recognition site for the restriction enzyme EcoRI was inserted into the primer of SEQ ID NO. 6 and the recognition site for the restriction enzyme XhoI was inserted into the primer of SEQ ID NO. 7. Subsequently, a nucleotide sequence (SEQ ID NO. 8) of the PCR product of approximately 1.4 kb obtained by PCR was examined.

[117]

[118] FVIIEcoRISS F (SEQ ID NO. 6):

[119] 5'-ccggaattcatggccaacgcgttcctggaggagctgcggccgggc-3'

[120] FVII#1XhoIAS R (SEQ ID NO. 7):

[121] 5'-ccgctcgagtcagcacacggccttcgtcgcgggaaatggggctcgcaggaggactcctgggc-3'

[122]

[123] In order to express the obtained PCR product under the control of CMV promoter, it was cloned into an animal cell expression vector pX0GC. Specifically, the PCR product was digested with the restriction enzymes, EcoRI and XhoI at 37°C for 2 hours, and applied to a PCR purification kit so as to obtain the cleaved DNA fragment. The DNA fragment was mixed with the pX0GC vector treated with the restriction enzymes EcoRI and XhoI, and cloned using T4 DNA ligase, thereby preparing an expression vector (pX0GC-FVII-ATKAVC) having a FacVII derivative-encoding polynucleotide, which contains a polynucleotide encoding the sequence ATKAVC (SEQ ID NO. 5) from 1 to 6 of the SOD1 sequence linked at the 3'-terminus of FacVII gene.

[124]

[125] **Example 2-2: Preparation of recombinant FacVII derivative-expressing vector, pX0GC-FVII-GGGGSC**

[126] A recombinant FacVII derivative-expressing vector pX0GC-FVII-GGGGSC, which contains a polynucleotide further having a polynucleotide encoding 6 amino acids (GGGGSC, SEQ ID NO. 10) at the 3'-terminus of FacVII gene included in the expression vector pX0GC-FVII prepared in Example 1, was prepared. To achieve this,

PCR was performed in the same manner as in Example 2-1, except using forward and reverse primers of SEQ ID NOs. 6 and 11, and a nucleotide sequence (SEQ ID NO. 12) of the PCR product of approximately 1.4 kb was examined. Subsequently, an expression vector (pX0GC-FVII-GGGGSC), which contains the FacVII derivative-encoding polynucleotide having a polynucleotide encoding GGGGSC (SEQ ID NO. 10) at the 3'-terminus of FacVII gene, was prepared in the same manner as in Example 2-1, except using the PCR product.

[127]

[128] FVII#2XhoIAS R (SEQ ID NO. 11):

[129] 5'-ccgctcgagtcagcaggagccgccgccgccgggaaatggggctcgcaggaggactcctgggc-3'

[130]

[131] **Example 2-3: Preparation of recombinant FacVII derivative-expressing vector, pX0GC-FVII-SOD1 1-149**

[132] A recombinant FacVII derivative-expressing vector pX0GC-FVII-SOD1 1-149, which contains a polynucleotide (SEQ ID NO. 15) further having a polynucleotide encoding 1 to 149 amino acids of the SOD1 (Superoxide dismutase 1) sequence at the 3'-terminus of FacVII gene included in the expression vector pX0GC-FVII prepared in Example 1, was prepared.

[133] In detail, the FacVII DNA sequence (SEQ ID NO. 3) was used as a template, and a FacVII forward primer (SEQ ID NO. 16) and a FacVII reverse primer (SEQ ID NO. 17) were used to perform PCR (95°C 1 minute denaturation; 30 cycles (95°C 60 seconds, 60°C 60 seconds and 68°C 90 seconds); 68°C 5 minutes). At this time, for easy cloning, the recognition site for the restriction enzyme EcoRI was inserted into the primer of SEQ ID NO. 16 and a partial sequence of 5'-terminus of SOD1 was contained in the primer of SEQ ID NO. 17. As a result, a first PCR fragment was obtained.

[134]

[135] FVIIEcoRISS F: 5'-ccggaattcatggtctcccaggccctcaggctcc-3' (SEQ ID NO. 16)

[136] FVIISODInfAS R: 5'-cggccttcgtcgcgggaaatggggctcgcaggag-3' (SEQ ID NO. 17)

[137]

[138] Next, SOD1 cDNA (RC200725, OriGene, USA) was used as a template, and an SOD1 forward primer (SEQ ID NO. 18) and an SOD1 reverse primer (SEQ ID NO. 19) were used to perform PCR (95°C 1 minute denaturation; 30 cycles (95°C 60 seconds, 60°C 60 seconds and 68°C 40 seconds); 68°C 5 minutes). At this time, for easy cloning, a partial sequence of 3'-terminus of FacVII was contained in the primer of SEQ ID NO. 18 and the recognition site for the restriction enzyme XhoI was inserted into the primer of SEQ ID NO. 19. As a result, a second PCR fragment was obtained.

[139]

[140] FVIISODInfSS F: 5'-gagccccatttcccgcgacgaaggccgtgtgcgt-3' (SEQ ID NO. 18)

[141] SODXhoIAS R: 5'-ccgctcgagtcaaattacaccacaagccaaacga-3' (SEQ ID NO. 19)

[142]

[143] The first and second PCR fragments thus obtained were used as a template and a FacVII forward primer (SEQ ID NO. 16) and a SOD1 reverse primer (SEQ ID NO. 19) were used to perform second PCR. Finally, a third PCR fragment was obtained (95°C 1 minute denaturation; 30 cycles (95°C 60 seconds, 60°C 60 seconds and 68°C 120 seconds); 68°C 5 minutes).

[144]

[145] In order to express the obtained third PCR product under the control of CMV promoter, it was cloned into an animal cell expression vector pX0GC. Specifically, the third PCR product was digested with the restriction enzymes, EcoRI and XhoI at 37°C for 2 hours, and applied to a PCR purification kit so as to obtain the cleaved DNA fragment. The DNA fragment was mixed with the pX0GC vector treated with the restriction enzymes EcoRI and XhoI, and cloned using T4 DNA ligase, thereby preparing an expression vector (pX0GC-FVII-SOD1 1-149) having a FacVII derivative-encoding polynucleotide, which contains a polynucleotide encoding the amino acids from 1 to 149 of the SOD1 sequence linked at the 3'-terminus of FacVII gene.

[146]

[147] **Example 2-4: Preparation of recombinant FacVII derivative-expressing vector, pX0GC-FVII-SOD1 IPRI**

[148] It was intended to insert a self-cleavage site of FacVII into the SOD1 gene included in the expression vector pX0GC-FVII-SOD1 1-149 prepared in Example 2-3. To achieve this, prepared was a recombinant FacVII derivative-expressing vector pX0GC-FVII-SOD1 IPRI which contains a polynucleotide (SEQ ID NO. 21) encoding an amino acid sequence (SEQ ID NO. 20) prepared by linking 1-149 amino acids of SOD1 mutated by replacement of 7 to 10 amino acids (VLKG) of SOD1 in 451-454 amino acids of FacVII-SOD1 1-149 (SEQ ID NO. 14) with IPRI, to the C-terminus of FacVII.

[149] In detail, the third PCR fragment obtained in Example 2-3 was used as a template and forward and reverse primers (SEQ ID NOs. 22 and 23) were used to perform PCR. Finally, a fourth PCR fragment was obtained (95°C 30 seconds denaturation; 18 cycles (95°C 30 seconds, 55°C 60 seconds and 68°C 9 minutes); 68°C 9 minutes).

[150] A nucleotide sequence (SEQ ID NO. 21) of the fourth PCR product of approximately 9 kb was examined.

[151]

[152] VIISOD1mutSS F: (SEQ ID NO. 22)

[153] 5'-cccgcgacgaaggccgtgtgcattccgaggatcgacggcccagtgcagggcatc-3'

[154] FVIISOD1mutAS R: (SEQ ID NO. 23)

[155] 5'-gatgccctgcactgggccgtcgatcctcggaatgcacacggccttcgtcgcggg-3'

[156]

[157] Subsequently, the fourth PCR product was digested with the restriction enzyme DpnI at 37°C for 1 hour to cleave a non-mutated sequence, and cloned by transformation into E. coli so as to prepare an expression vector (pX0GC-FVII-SOD1 IPRI) which contains a polynucleotide encoding a FacVII derivative having 1-149 amino acids of the mutated SOD1 at the C-terminus of FacVII.

[158]

[159] **Example 2-5: Preparation of recombinant FacVII derivative-expressing vector, pX0GC-FVII-SOD1 1-25 IPRI**

[160] Prepared was a recombinant FacVII derivative-expressing vector pX0GC-FVII-SOD1 1-25 IPRI which contains a polynucleotide (SEQ ID NO. 25) encoding an amino acid sequence (SEQ ID NO. 24) prepared by linking 1-25 amino acids of SOD1 mutated by replacement of 7 to 10 amino acids (VLKG) of SOD1 with IPRI to the C-terminus of FacVII.

[161] In detail, the expression vector pX0GC-FVII-SOD1 IPRI prepared in Example 2-4 was used as a template, and forward and reverse primers (SEQ ID NOs. 16 and 26) were used to perform PCR (95°C 1 minute denaturation; 30 cycles (95°C 60 seconds, 60°C 60 seconds and 68°C 90 seconds); 68°C 5 minutes). Finally, a fifth PCR fragment was obtained.

[162]

[163] SOD1-25XhoIAS R: 5'-ccgctcgagtcaactttccttctgctcgaaattg-3'(SEQ ID NO. 26)

[164]

[165] Subsequently, the fifth PCR product was digested with the restriction enzymes EcoRI and XhoI at 37°C for 2 hour, and applied to a PCR purification kit so as to obtain the cleaved DNA fragment. The DNA fragment was mixed with the pX0GC vector treated with the restriction enzymes EcoRI and XhoI, and cloned using T4 DNA ligase, thereby preparing an expression vector (pX0GC-FVII-SOD1 1-25 IPRI) containing a polynucleotide encoding a FacVII derivative having 1 to 25 amino acids of the mutated SOD1 sequence linked at the C-terminus of FacVII gene.

[166]

[167] **Example 2-6: Preparation of recombinant FacVII derivative-expressing vector, pX0GC-FVII-SOD1 1-90 IPRI**

[168] Prepared was a recombinant FacVII derivative expression vector pX0GC-FVII-SOD1 1-90 IPRI which contains a polynucleotide (SEQ ID NO. 28) encoding an amino acid

sequence (SEQ ID NO. 27) prepared by linking 1-90 amino acids of SOD1 mutated by replacement of 7 to 10 amino acids (VLKG) of SOD1 with IPRI to the C-terminus of FacVII.

[169] In detail, the expression vector pX0GC-FVII-SOD1 IPRI prepared in Example 2-4 was used as a template, and forward and reverse primers (SEQ ID NOs. 16 and 29) were used to perform PCR (95°C 1 minute denaturation; 30 cycles (95°C 60 seconds, 60°C 60 seconds and 68°C 100 seconds); 68°C 5 minutes). Finally, a fifth PCR fragment was obtained.

[170]

[171] SOD1-90XhoIAS R: 5'-ccgctcgagtcagtcagcagtcacattgcccaag-3'(SEQ ID NO. 29)

[172]

[173] Subsequently, the fifth PCR product was digested with the restriction enzymes EcoRI and XhoI at 37°C for 2 hour, and applied to a PCR purification kit so as to obtain the cleaved DNA fragment. The DNA fragment was mixed with the pX0GC vector treated with the restriction enzymes EcoRI and XhoI, and cloned using T4 DNA ligase, thereby preparing an expression vector (pX0GC-FVII-SOD1 1-90 IPRI) containing a polynucleotide encoding a FacVII derivative having 1 to 90 amino acids of the mutated SOD1 sequence linked at the C-terminus of FacVII gene.

[174]

[175] **Example 3: Expression of FacVII derivatives**

[176] A variety of FacVII derivatives were expressed using each expression vector prepared in Example 2.

[177]

[178] **Example 3-1: Expression of FacVII derivatives in 293F cell line**

[179] An expression vector having nothing at the C-terminus of FacVII (control), and expression vectors (pX0GC-FVII-SOD1 1-149, pX0GC-FVII-SOD1 1-25 IPRI, pX0GC-FVII-SOD1 1-90 IPRI, pX0GC-FVII-ATKAVC or pX0GC-FVII-GGGGSC), each expression vector having a polynucleotide prepared by fusion of SOD1 1-149 sequence, mutated SOD1 1-25 sequence, mutated SOD1 1-90 sequence, SOD1 1-6 sequence, or GGGGSC sequence at the C-terminus of FacVII, were introduced into Freestyle™ 293 F cell line (Invitrogen, cat. no. R79007) to prepare each transformant. Different FacVII derivatives were expressed from each transformant.

[180]

[181] To achieve this, the 293F cell line was sub-cultured every other day while it cultured at 37°C and 8% CO_2 with shaking at a speed of 120 rpm or higher. When the number of cultured cells reached 10 x 10^5 cells/ml and the viability was 85% or more, each expression vector prepared in Example 2 was introduced thereto so as to obtain transformants. In detail, 500 μl of Freestyle™ max reagent, 500 μg of each expression

vector, and 10 ml of Optipro™ SFM (Invitrogen, cat. no. 12309-050) were added to 500 ml of Freestyle™ 293 expression medium (Invitrogen, cat. no. 12338-018) having 10 x 10^5 cells/ml of cells, and mixed and left at room temperature for 10 minutes for introduction of each expression vector into 293F cell line. Thereafter, 50 μg of vitamin K essential for FacVII activation was added thereto, and cultured at 37°C and 8% CO_2 with shaking at a speed of 120 rpm or higher for 3 days. After completion of the culture, the cells were centrifuged at 3000 rpm for 5 minutes to obtain the supernatants. Each FacVII derivative included in the supernatant was collected and purified. Subsequently, Western blotting was performed using anti-FacVII antibody to detect each expressed FacVII derivative (FIGs. 1a to 1c).

[182]

[183] FIG. 1a is a photograph showing the result of Western blot analysis of FacVII-ATKAVC expressed in 293F cell line, in which M is a size marker (Prestained protein ladder, fermentas), Lane 1 is the result of Western blot analysis of FacVII-ATKAVC under reducing conditions, and Lane 2 is the result of Western blot analysis of FacVII-ATKAVC under non-reducing conditions. As shown in FIG. 1a, approximately 20 to 30% of a dimeric form was detected under non-reducing conditions, and no dimeric form was detected under reducing conditions.

[184] Further, FIG. 1b is a photograph showing the result of Western blot analysis of a control group and FacVII-GGGGSC expressed in 293F cell line, and FIG. 1c is a photograph showing the result of Western blot analysis showing the molecular weight difference of FacVII-ATKAVC and FacVII-SOD1 1-149 expressed in 293F cell line.

[185] As shown in FIGs. 1b and 1c, it was found that a variety of FacVII derivatives can be normally produced in 293F cell line.

[186]

[187] **Example 3-2: Expression of FacVII derivative (pX0GC-FVII-ATKAVC) in CHO cell line**

[188] The expression vector pX0GC-FVII-ATKAVC prepared in Example 2-1 was introduced into DG44/CHO cell line (CHO/dhfr-) that is deficient in DHFR to show incomplete DNA synthesis (Urlaub et al., Somat. Cell. Mol. Genet., 12, 555-566, 1986) to obtain a transformant, and FacVII-ATKAVC derivative was expressed from the transformant.

[189] In detail, the DG44/CHO cell line was cultured to reach 80 to 90% confluence, and the cells were washed with Opti-MEM (Gibco, cat. No. 51985034) three times.

[190] On the other hand, a mixture of 3 ml of Opti-MEM and 5 μg of expression vector pX0GC-FVII-ATKAVC, and a mixture of 3 ml of Opti-MEM and 20μl of lipofectamine (Gibco, cat. no. 18324-012) were left at room temperature for 30 minutes, respectively. Subsequently, the mixtures were mixed, and added to the cultured

DG44/CHO cell line. Then, the cells were cultured at 37°C and 5% CO_2 for approximately 18 hours, resulting in introduction of the expression vector pX0GC-FVII-ATKAVC into DG44/CHO cell line. Subsequently, the cultured cells were washed with 10% FBS-supplemented DMEM-F12 (Gibco, cat. no. 11330) three times, and then the medium was added thereto, followed by cultivation for 48 hours. The cultured cells were detached by trypsin treatment, and they were inoculated into MEM-α medium (WELGENE, cat. no. LM008-02)) containing 10% FBS and 1 mg/ml of G418 (Cellgro, cat. no. 61-234 -RG) without selection medium (HT supplement (Hypoxanthine-Thymidine)). Until the transformed cells survived to form colonies, the medium was replaced with the selection medium every 2 or 3 days. Thus, the transformed cells were selected from the separated cells. At this time, in order to increase the expression level of FacVII-ATKAVC derivative in the selected transformed cells, 10 nM MTX (Sigma, cat. no. M8407) was added to the selection medium to gradually increase the concentration, and 2 to 3 weeks later, the content of MTX was increased up to 30 nM.

[191] Furthermore, in order to reduce heterogeneity of the transformed cells, a limiting dilution method was performed to obtain single clones. In detail, the transformed cells were diluted to a ratio of 0.7 cell in each well of a 96-well plate, and cultured for 2 to 3 weeks to examine whether single clones were observed. When cluster formation of single clones were observed in wells, the single clones were transferred to a 24-well plate, and cell growth rate of each clone and expression level of FacVII derivative were analyzed by ELISA so as to select a clone showing the highest expression level of FacVII derivative. It was designated as "HMF709", and deposited at the Korean Collection for Type Culture, Korea Research Institute of Bioscience and Biotechnology (111 Gwahangno, Yuseong-gu, Daejeon, Korea) on Sep. 23, 2011 under accession number "KCTC12022BP".

[192]

[193] **Example 4: Purification of FacVII-ATKAVC**

[194] The transformant prepared in Example 3-2 was cultured to express FacVII-ATKAVC, and the culture solution was centrifuged at 3000 rpm for 5 minutes to obtain a supernatant.

[195] The supernatant was filtered using a 0.2 μm microfiltration membrane, and 0.6 M ammonium sulfate was added thereto, and the mixture was applied to a butyl HP column. Elution was performed using a concentration gradient buffer solution (20 mM Tris-HCl pH 7.5) containing 0.6-0 M ammonium sulfate to obtain an active fraction containing FacVII-ATKAVC.

[196] The buffer solution of the obtained active fraction was replaced with a 10 mM sodium phosphate buffer solution (pH 7.0), which was applied to a Heparin HP column

and eluted using a 0-1.0 M NaCl concentration gradient buffer solution (10 mM sodium phosphate, pH 7.0) so as to obtain an active fraction containing FacVII-ATKAVC.

[197] The active fraction was concentrated, and applied to a Superdex75 column, and then eluted using 150 mM NaCl 20 mM Tris-HCl (pH 7.5) buffer solution so as to obtain an active fraction containing FacVII-ATKAVC. The buffer solution of the obtained active fraction was replaced with a 2 mM benzamidine 20 mM Tris-HCl (pH 7.5) buffer solution, which was applied to a Q FF column. Then, washing (2 mM benzamidine 0.2 M NaCl 20 mM Tris-HCl (pH 8.0) buffer), re-equilibration (2 mM benzamidine 0.1 M NaCl 20 mM Tris-HCl (pH 8.0) buffer), and concentration-gradient elution (2 mM benzamidine 25 mM NaCl 35 mM $CaCl_2$, 20 mM Tris-HCl (pH 8.0) buffer) were performed to purify FacVII-ATKAVC.

[198] The purity of the purified FacVII-ATKAVC was examined by SDS PAGE (FIG. 2). FIG. 2 is a photograph showing the result of electrophoresis of the purified FacVII-ATKAVC, in which M is a size marker, Lane 1 is FacVIII under reducing conditions, Lane 2 is of FacVII-ATKAVC under reducing conditions, Lane 3 is FacVII under non-reducing conditions, and Lane 4 is FacVII-ATKAVC under non-reducing conditions.

[199]

[200] **Example 5: Preparation of conjugates of FacVII-ATKAVC and PEG**

[201] FacVII-ATKAVC purified in Example 4 was conjugated with PEG having different molecular weights to prepare conjugates.

[202]

[203] **Example 5-1: Preparation of FacVII-ATKAVC-40 kDa PEG conjugate**

[204] For PEGylation of the C-terminus of FacVII-ATKAVC with 40 kDa mPEG-maleimide (NOF, Japan), FacVII-ATKAVC (1 mg/ml) and 40 kDa mPEG-maleimide were mixed at a molar ratio of 1:20 in the presence of a 100 mM phosphate buffer solution (pH 5.5), and a reducing agent, 2 mM triarylphosphine was added thereto, and reacted at 25°C for 2 hours. As a result, mono-PEGylated FacVII-ATKAVC (FacVII-ATKAVC-40k PEG conjugate) was prepared (FIG. 3). FIG. 3 is a photograph showing the result of electrophoresis of a conjugate of FacVII-ATKAVC and PEG, in which M is a size marker, Lane 1 is FacVII-ATKAVC-40 kDa PEG conjugate under non-reducing conditions, and Lane 2 is FacVII-ATKAVC-5 kDa PEG conjugate under non-reducing conditions.

[205]

[206] **Example 5-2: Preparation of FacVII-ATKAVC-5 kDa PEG conjugate**

[207] PEGylation of the C-terminus of FacVII-ATKAVC with aldehyde-5 kDa PEG-maleimide (NOF, Japan) was performed in the same manner as in Example 5-1, except

using aldehyde-5 kDa PEG-maleimide instead of 40 kDa mPEG-maleimide so as to prepare PEGylated FacVII-ATKAVC (FacVII-ATKAVC-5 kDa PEG conjugate) (FIG. 3).

[208]

[209] **Example 5-3: Preparation of FacVIIa-ATKAVC-PEG-Fc complex**

[210] To link the aldehyde reactive group of maleimide-10 kDa PEG-aldehyde (NOF, Japan) to the N-terminus of immunoglobulin Fc region, the immunoglobulin Fc region and maleimide-10 kDa PEG-aldehyde were mixed at a molar ratio of 1:1 in the presence of 100 mM phosphate buffer solution (pH 6.0), and a reducing agent, 20 mM $Na\text{-}CNBH_3$ was added under the condition of a protein concentration of 10 mg/ml. The mixture was reacted at low temperature (4~8°C) for 2 hours. In order to obtain a mono-PEGylated immunoglobulin Fc region (maleimide-10 kDa PEG-Fc), cation exchange chromatography was performed using Source 15Q, and elution was performed in a 20 mM Tris buffer solution (pH 7.5) using a sodium chloride concentration gradient.

[211] On the other hand, the C-terminus of FacVII-ATKAVC was reduced in a 10 mM Glycil-Glycine buffer solution (pH 5.5) using a reducing agent, 0.5~2 mM triphenylphosphine-3,3',3"-trisulfonic trisodium salt hydrate at room temperature for 2 hours.

[212] The C-terminus-reduced FacVII-ATKAVC and the mono-PEGylated immunoglobulin Fc region (maleimide-10kDa PEG-Fc) were mixed at a molar ratio of 1:4~1:20, and reacted at room temperature for 2 hours in the presence of 50 mM Tris buffer solution (pH 7.5) at a total protein concentration of 1~2 mg/ml. First, cation exchange chromatography was performed using Source 15Q, and a FacVII-ATKAVC-10k PEG-Fc complex was eluted in a 20 mM Tris buffer solution (pH 7.5) using a sodium chloride concentration gradient.

[213]

[214] For activation of FacVII in the FacVII-ATKAVC-PEG-Fc complex, solution reaction was performed in a 0.1 M Tris-HCl buffer solution (pH 8.0) at approximately 4 mg/ml based on FacVII at low temperature (4~8°C) for 18 hours.

[215] Size exclusion chromatography was performed using Superdex200 and a 10 mM Glycil-Glycine buffer solution (pH 5.5) so as to purify final FacVIIa-ATKAVC-PEG-Fc.

[216] The purity of the purified FacVIIa-ATKAVC-PEG-Fc was examined by SDS PAGE and Western blotting (FIG. 4). FIG. 4a is a photograph showing the result of electrophoresis of the purified FacVIIa-ATKAVC-PEG-Fc conjugate, in which M is a size marker, Lane 1 is FacVIIa-ATKAVC-PEG-Fc under reducing conditions, and Lane 2 is FacVIIa-ATKAVC-PEG-Fc under non-reducing conditions. FIG. 4b is a photograph showing the result of Western blot analysis of the purified FacVIIa-ATKAVC-PEG-Fc,

in which Lane 1 is FacVIIa-ATKAVC-PEG-Fc under reducing conditions, and Lane 2 is FacVIIa-ATKAVC-PEG-Fc under non-reducing conditions.

[217]

[218] **Example 6: In vitro activity (EC_{50}) of FacVII and FacVII-ATKAVC**

[219] In order to determine in vitro activities of FacVII and FacVII-ATKAVC, a commercial kit (Chromogenix, COASET) was used to perform chromogenic assay. The activity assay was performed in accordance with the European Pharmacopoeia "2.7.10. ASSAY OF HUMAN COAGULATION FACTOR VII".

[220] The diluted FacVII and FacVII-ATKAVC are activated by thromboplastin and Ca^{2+} ions. FX is activated to FXa by the activated FacVIIa and FacVIIa-ATKAVC, and a substrate S-2765 (N-a-Cbo-D-Arg-Gly-Arg-pNA) is hydrolyzed and dissociated into a peptide and a chromophoric group pNA by the activated FXa. The absorbance of the dissociated pNA at 405 nm was monitored to determine the in vitro activities of FacVIIa and FacVIIa-ATKAVC.

[221] Changes in absorbance according to the concentrations of FacVII and FacVII-ATKAVC were examined by regression analysis using a 4-parameter model of Softmax Pro 4.0 program, and the activities between two substances were compared using the obtained EC_{50} values.

[222] The test results (FIG. 5 and Table 1) showed that the in vitro activity of FacVII-ATKAVC shows a titer equivalent to or higher than that of the native FacVII.

[223] Table 1

[Table 1]

EC_{50} comparison between FacVII and FacVII-ATKAVC

	Lot. No.	EC_{50} (ng/mL) (Relative activity, %)	
		Run 1	Run 2
FacVII	B13161-PKC251	1.62 (100)	1.67 (100)
FacVII-ATKAVC	B13090-PKI111	1.36 (119)	1.16 (143)

[224] As shown in Table 1 and FIG. 5, FacVII and FacVII-ATKAVC were found to exhibit equivalent in vitro activities, indicating that the FacVII or FacVII derivative of the present invention has an activity equivalent to that of native form, and addition of a peptide linker to the C-terminus does not affect its activity.

[225]

[226] **Example 7: In vitro activity (EC_{50}) of FacVII-ATKAVC and**

FacVII-ATKAVC-40 kDa PEG

[227] In order to examine the activity according to site-specific conjugation, in vitro activities of FacVII-40 kDa PEG, FacVII-ATKAVC, and C-terminal PEGylated FacVII-ATKAVC-40 kDa PEG were determined. A commercial kit (Chromogenix, COASET) was used to perform chromogenic assay, and the method was performed in the same manner as in Example 6. Changes in absorbance according to the concentrations of test samples were examined using a 4-parameter model of Softmax Pro 4.0 program, and the relative activities after PEGylation were examined using the obtained EC_{50} values.

[228] The test results (Table 2) showed that the in vitro activity of N-terminal PEGylated FacVII-40 kDa PEG shows a titer of approximately 11%, compared to FacVII, and in vitro activity of C-terminal PEGylated FacVII-ATKAVC-40 kDa PEG shows a titer of approximately 29%, compared to FacVII-ATKAVC.

[229]

[230] Table 2

[Table 2]

EC_{50} comparison between FacVII-ATKAVC and FacVII-ATKAVC-40 kDa PEG

	Lot. No.	EC_{50} (ng/mL) (Relative activity, %)
FacVII	B13160-PJE261	1.38 (100)
FacVI-40 kDa PEG	B13161-PKI291	12.17 (11.3)
FacVII-ATKAVC	B13190-PKI261	1.75 (100)
FacVII-ATKAVC-40 kDa PEG	B13161-PKI292	6.06 (28.9)

[231] The activity of the conjugate of PEG and FacVII derivative of the present invention showed a titer of approximately 29%, compared to FacVII derivative. In contrast, the activity of the conjugate of PEG and FacVII showed a titer of approximately 11%, compared to FacVII. These results indicate that the C-terminal PEGylated FacVII-ATKAVC-40 kDa PEG maintains approximately 2.5 times higher relative activity, compared to EC_{50} of the N-terminal PEGylated FacVII-40 kDa PEG, and the activity of FacVII can be highly maintained by site-specific conjugation using ATKAVC.

[232]

[233] **Example 8: In vitro activity of FacVIIa-ATKAVC-PEG-Fc**

[234] In vitro activity of FacVIIa-ATKAVC-PEG-Fc was determined using a commercial kit (StaclotVIIa-rTF, Stago) and international standard NIBSC Factor VIIa (656 IU/vial, Code No. 07/228) as a standard material. This method is based on coagulation by specific reaction of rsTF (recombinant soluble tissue factor) and Factor VIIa. NIBSC Factor VIIa, FacVIIa-ATKAVC, and FacVIIa-ATKAVC-PEG-Fc were diluted with FacVII-deficient human plasma at a ratio of 1:1, and reacted with a mixture of rsTF and phospholipid for approximately 180 seconds. Thereafter, 25 mM $CaCl_2$ was added thereto to measure the time of coagulation. As the amount of Factor VIIa increases, the coagulation time becomes shorter.

[235] In order to calculate a specific activity (IU/mg) of FacVIIa-ATKAVC and FacVIIa-ATKAVC-PEG-Fc, potencies (IU/mL) of FacVIIa-ATKAVC and FacVIIa-ATKAVC-PEG-Fc relative to potency (IU/mL) of NIBSC Factor VIIa were first analyzed using PLA 2.0. Thereafter, the calculated potency (IU/mL) was divided by the protein concentration (mg/mL) to calculate the specific activity.

[236] The test results (Table 3) showed that in vitro activity of FacVIIa-ATKAVC-PEG-Fc was approximately 20632 IU/mg, indicating that it maintains approximately 45% activity, compared to FacVIIa-ATKAVC.

[237]

[238] Table 3

[Table 3]

In vitro activity of FacVIIa-ATKAVC-PEG-Fc

	Lot. No.	Potency (IU/mL)	Specific activity (IU/mg as FacVIIa) (Residual activity, %)
FacVIIa-ATKAVC	B13090-PLD111	16704.0	46143.6 (100)
Fac VIIa-ATKAVC-PEG-Fc	B13099-LLA041	1993.7	20632.2 (44.7)

[239]

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : **LEE, Kwan Sun**
Hanmi Pharm. Co., Ltd.
893-3 Hajeo-ri, Paltan-myun, Hwaseong-si, Gyeonggi-do 445-958
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: **HMF709** **(CHO cell line (dhfr-))**	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **KCTC 12022BP**
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: [x] a scientific description [ ] a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **September 23, 2011.**	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: **Korean Collection for Type Cultures** Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) 111 Gwahangno, Yuseong-gu, Daejeon 305-806 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s): K. S. Bae BAE, Kyung Sook, Director Date: **September 29, 2011**

Form BP/4 (KCTC Form 17) sole page

Claims

[Claim 1] A derivative of FacVII or its active form FacVIIa, comprising an amino acid sequence (SEQ ID NO. 4) of blood coagulation factor VII (Factor VII, FacVII) and a peptide linker linked at the C-terminus thereof.

[Claim 2] The derivative according to claim 1, wherein the C-terminal amino acid residue of the peptide linker is a cysteine.

[Claim 3] The derivative according to claim 1, wherein the peptide linker is a partial sequence of SOD1 (Superoxide dismutase).

[Claim 4] The derivative according to claim 3, wherein the partial sequence of SOD1 is mutated by replacing of VLKG (valine-leucine-lysine-glycine) within the sequence with a self-cleavage site sequence IPRI (isoleucine-proline-arginine-isoleucine).

[Claim 5] The derivative according to claim 1, wherein the derivative is composed of an amino acid sequence selected from the group consisting of SEQ ID NOs. 9, 13, 14, 20, 24, and 27.

[Claim 6] A polynucleotide encoding the derivative of FacVII or its active form FacVIIa of any one of claims 1 to 5.

[Claim 7] The polynucleotide according to claim 6, wherein the polynucleotide is composed of a nucleotide sequence selected from the group consisting of SEQ ID NOs. 8, 12, 15, 21, 25 and 28.

[Claim 8] An expression vector comprising the polynucleotide of claim 6.

[Claim 9] The expression vector according to claim 8, wherein the vector is selected from the group consisting of pX0GC-FVII-ATKAVC, pX0GC-FVII-GGGGSC, pX0GC-FVII-SOD1 1-149, pX0GC-FVII-SOD1 IPRI, pX0GC-FVII-SOD1 1-25 IPRI, and pX0GC-FVII-SOD1 1-90 IPRI.

[Claim 10] A transformant comprising the expression vector of claim 8.

[Claim 11] The transformant according to claim 10, wherein the transformant is identified by accession number KCTC12022BP.

[Claim 12] A method for preparing a FacVII derivative, comprising the steps of:
(i) culturing the transformant of claim 10 so as to obtain a culture solution; and
(ii) recovering the FacVII derivative from the culture solution.

[Claim 13] The method according to claim 12, further comprising the step of activating the recovered FacVII derivative.

[Claim 14] A conjugate of FacVII or its active form FacVIIa, wherein a non-peptidyl polymer capable of extending the blood half-life is linked to

the peptide linker of the derivative of FacVII or its active form FacVIIa of any one of claims 1 to 5.

[Claim 15] The conjugate according to claim 14, wherein the non-peptidyl polymer is linked to the C-terminus of the derivative of FacVII or its active form FacVIIa .

[Claim 16] The conjugate according to claim 14, wherein the non-peptidyl polymer has one or more reactive group(s) selected from the group consisting of an aldehyde group, a propionaldehyde group, a butyraldehyde group, a maleimide group, ortho pyridyl disulfide, thiol and succinimide derivatives.

[Claim 17] The conjugate according to claim 14, wherein the succinimide derivative is succinimidyl propionate, succinimidyl carboxymethyl, hydroxy succinimidyl or succinimidyl carbonate.

[Claim 18] The conjugate according to claim 14, wherein the non-peptidyl polymer has two or three reactive ends.

[Claim 19] The conjugate according to claim 14, wherein the non-peptidyl polymer has a maleimide reactive group or an aldehyde reactive group at both ends, respectively.

[Claim 20] The conjugate according to claim 14, wherein the non-peptidyl polymer is selected from the group consisting of polyethylene glycol, polypropylene glycol, ethylene glycol-propylene glycol non-peptidyl copolymers, polyoxyethylated polyols, polyvinyl alcohols, polysaccharides, dextrans, polyvinyl ethyl ethers, biodegradable polymers, lipid non-peptidyl polymers, chitins, hyaluronic acids, and a combination thereof.

[Claim 21] A complex of FacVII or its active form FacVIIa that is composed of the FacVII or FacVIIa derivative of any one of claims 1 to 5 linked to an immunoglobulin Fc region via a non-peptidyl polymer.

[Claim 22] The complex according to claim 21, wherein the non-peptidiyl polymer is selected from the group consisting of polyethylene glycol, polypropylene glycol, ethylene glycol-propylene glycol copolymers, polyoxyethylated polyols, polyvinyl alcohols, polysaccharides, dextrans, polyvinyl ethyl ethers, biodegradable polymers, lipid polymers, chitins, hyaluronic acids, and a combination thereof.

[Claim 23] The complex according to claim 21, wherein the non-peptidyl polymer has one, two or three reactive end(s).

[Claim 24] The complex according to claim 21, wherein the non-peptidyl polymer has a maleimide reactive group or an aldehyde reactive group at both

ends, respectively.

[Claim 25] The complex according to claim 21, wherein the immunoglobulin Fc region is composed of one to four domains selected from the group consisting of CH1, CH2, CH3 and CH4 domains.

[Claim 26] The complex according to claim 21, wherein the immunoglobulin Fc region further comprises a hinge region.

[Claim 27] The complex according to claim 21, wherein the immunoglobulin Fc region is derived from IgG, IgA, IgD, IgE, or IgM.

[Claim 28] The complex according to claim 21, wherein the immunoglobulin Fc region is an IgG4 Fc region.

[Claim 29] The complex according to claim 21, wherein the immunoglobulin Fc region is a human aglycosylated IgG4 Fc region.

[Claim 30] A method for preparing a FacVIIa complex, comprising the steps of:
(i) covalently linking a non-peptidyl polymer having a succinimide derivative or aldehyde reactive group at its one end to the amine group of immunoglobulin Fc at pH 5.0 ~ pH 9.0;
(ii) recovering a conjugate that includes the immunoglobulin Fc region covalently linked with the non-peptidyl polymer at the amine group, from the reaction mixture of step (i);
(iii) covalently linking the C-terminal thiol group of the FacVII derivative of any one of claims 1 to 5 to the other end of the non-peptidyl polymer in the recovered conjugate so as to produce a FacVII complex having the immunoglobulin Fc region and the FacVII derivative at each end of the non-peptidyl polymer; and
(iv) activating the FacVII complex produced in step (iii) so as to produce a FacVIIa complex having FacVIIa and the immunoglobulin Fc region linked via the non-peptidyl polymer.

[Claim 31] A method for preparing a FacVIIa complex, comprising the steps of:
(i) covalently linking a non-peptidyl polymer having a succinimide derivative or aldehyde reactive group at its one end to the amine group of immunoglobulin Fc at pH 5.0 ~ pH 9.0;
(ii) recovering a conjugate that includes the immunoglobulin Fc region covalently linked with the non-peptidyl polymer at the amine group, from the reaction mixture of step (i); and
(iii) covalently linking the C-terminal thiol group of the FacVIIa derivative of any one of claims 1 to 5 to the other end of the non-peptidyl polymer in the recovered conjugate so as to produce a FacVIIa complex having the immunoglobulin Fc region and the FacVIIa

derivative at each end of the non-peptidyl polymer.

[Claim 32] A method for preparing a FacVIIa conjugate, comprising the steps of:
(i) covalently linking a non-peptidyl polymer having a reactive group selected from the group consisting of an aldehyde group, a propionaldehyde group, a butyraldehyde group, a maleimide group, ortho pyridyl disulfide, thiol and succinimide derivatives to the C-terminal thiol group of the FacVII derivative of any one of claims 1 to 5;
(ii) recovering the FacVII conjugate prepared by covalently linking the non-peptidyl polymer to the FacVII derivative; and
(iii) activating the recovered FacVII conjugate so as to produce a FacVIIa conjugate having the non-peptidyl polymer linked to the FacVIIa region.

[Claim 33] A method for preparing a FacVIIa conjugate, comprising the steps of:
(i) covalently linking a non-peptidyl polymer having a reactive group selected from the group consisting of an aldehyde group, a propionaldehyde group, a butyraldehyde group, a maleimide group, ortho pyridyl disulfide, thiol and succinimide derivatives to the C-terminal thiol group of the FacVIIa derivative of any one of claims 1 to 5 ; and
(ii) recovering the FacVIIa conjugate prepared by covalently linking the non-peptidyl polymer to the FacVIIa derivative.

[Claim 34] A FacVIIa complex that is composed of an immunoglobulin Fc region linked to the other end of the non-peptidyl polymer in the recovered FacVIIa conjugate of claim 32 or 33.

[Claim 35] The method according to any one of claims 30 to 33, wherein activation of the FacVII derivative, FacVII conjugate, or FacVII complex is performed by on-column activation or in-solution activation.

[Claim 36] The method according to any one of claims 30 to 33, wherein the non-peptidyl polymer is polyethylene glycol.

[Claim 37] A FacVIIa conjugate or FacVIIa complex that is prepared by the method of any one of claims 30 to 33.

[Claim 38] A complex of FacVII or its active form FacVIIa that is composed of the FacVII derivative or the FacVIIa derivative linked with a carrier via a non-peptidyl polymer selected from the group consisting of polyethylene glycol, polypropylene glycol, ethylene glycol-propylene glycol copolymers, polyoxyethylated polyols, polyvinyl alcohols, polysaccharides, dextrans, polyvinyl ethyl ethers, biodegradable polymers, lipid polymers, chitins, hyaluronic acids, and a combination thereof.

[Claim 39] The complex according to claim 14, wherein one end of the non-peptidyl polymer constituting the FacVII conjugate or FacVIIa conjugate is linked to a carrier selected from the group consisting of antibody, albumin, and transferrin.

[Claim 40] The complex according to claim 38, wherein the carrier is selected from the group consisting of antibody, albumin, and transferrin.

[Claim 41] The complex according to claim 38, wherein the non-peptidyl polymer has one, two or three reactive end(s).

[Claim 42] The complex according to claim 38, wherein the non-peptidyl polymer has a maleimide reactive group or an aldehyde reactive group at both ends, respectively.

[Claim 43] A pharmaceutical composition for preventing or treating hemophilia, comprising the FacVII derivative or the FacVIIa derivative of any one of claims 1 to 5, the FacVII conjugate or the FacVIIa conjugate of any one of claims 14 to 20, the FacVII complex or the FacVIIa complex of any one of claims 21 to 29, or the FacVIIa conjugate or the FacVIIa complex of claim 37, as an active ingredient.

[Claim 44] A pharmaceutical composition for promoting blood coagulation, comprising the FacVII derivative or the FacVIIa derivative of any one of claims 1 to 5, the FacVII conjugate or the FacVIIa conjugate of any one of claims 14 to 20, the FacVII complex or the FacVIIa complex of any one of claims 21 to 29, or the FacVIIa conjugate or the FacVIIa complex of claim 37, as an active ingredient.

[Claim 45] A method for preventing or treating hemophilia, comprising the step of administering to a subject a therapeutically effective amount of the pharmaceutical composition for preventing or treating hemophilia of claim 43.

[Claim 46] A method for promoting blood coagulation, comprising the step of administering to a subject a therapeutically effective amount of the pharmaceutical composition for promoting blood coagulation of claim 44.