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NOTICE OF ENTITLEMENT

I ~~(We)~~ William G Thurman M.D.

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the applicant and nominated person in respect of an application for a patent for an invention entitled "Truncated Tissue Factor and FVIIa or FVII Activator for Blood Coagulation"

filed under Australian Application No. 43729/93 , state the following:

PART 1 - *Must be completed for all applications.*

The person(s) nominated for the grant of the patent

~~is (are) the actual inventor(s)~~

or

X has, for the following reasons, gained entitlement from the actual inventor(s)

The nominated person is the assignee of the invention from the actual inventors.

PART 2 - ~~*Must be completed if the application is a Convention application.*~~

The person(s) nominated for the grant of the patent is (are):

the applicant(s) of the basic application(s) listed on the patent request form

or

entitled to rely on the basic application(s) listed on the patent request form by reason of the following:

The basic application(s) listed on the request form is (are) the first application(s) ~~made~~ in a Convention country in respect of the invention.

PART 3 - *Must be completed if the application was made under the PCT and claims priority.*

The person(s) nominated for the grant of the patent is (are):

~~the applicant(s) of the application(s) listed in the declaration under Article 8 of the PCT~~

or

X entitled to rely on the application(s) listed in the declaration under Article 8 of the PCT by reason of the following:

~~xxxx xxxxxxxxxxxx xxxxxxxx xxxx xxxx xxxx xxxx xxxx xxxx xxxx xxxx xxxx~~ The nominated person is the assignee of the basic application from the said actual inventor(s)

The basic application(s) listed in the declaration made under Article 8 of the PCT is (are) the first application(s) made in a Convention country in respect of the invention.

Dated this 12th day of October 19 94

Signed William G Thurman M.D. Status President

Signatory's Name William G. Thurman, M.D.

F.B. RICE & CO PATENT ATTORNEYS

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TRUNCATED TISSUE FACTOR AND FVIIA OR FVII ACTIVATOR FOR BLOOD COAGULATION

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(56) Prior Art Documents
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(57) Claim

1. A composition for treatment of patients with prolonged or excessive bleeding, comprising (i) a soluble truncated tissue factor (tTF) comprised of the extracellular domain, or a functionally-equivalent fragment thereof, of wild-type tissue factor (TF), said truncated tissue factor characterised by its inability to bind to phospholipids and its inability to support conversion of factor VII to factor VIIa and (ii) a substance selected from factor VIIa and activators of endogenous factor VII other than wild-type tissue factor, said substance effective to produce a plasma level of factor VIIa which in combination with said truncated tissue factor will control or stop said prolonged or excessive bleeding.

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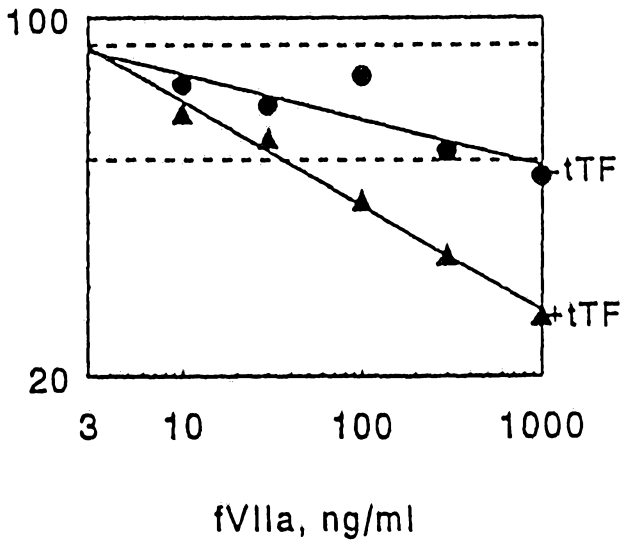

(57) Abstract

It has been discovered that it is possible to administer truncated tissue factor, not having the transmembrane region, (tTF) in combination with factor VIIa (FVIIa) or an activator of endogenous factor VII to treat bleeding disorders such as those resulting from hemophilia or cirrhosis of the liver. The tTF is administered to produce up to 10 μg tTF/ml of plasma. The FVIIa or FVII activator is administered to produce levels of between 40 ng VIIa/ml and 700 ng FVIIa/ml of plasma. The effective dosages of both tTF and VIIa/factor VII activator are significantly and surprisingly less than the administration of either alone to stop bleeding. Examples demonstrate safety and efficacy in normal and hemophilic dogs.

TRUNCATED TISSUE FACTOR AND FVIIa OR FVII ACTIVATOR FOR BLOOD COAGULATION

Technical Field

The present invention relates to a composition and method for treatment of bleeding.

Background of the Invention

The United States government has certain rights in this invention by virtue of grant R01 HL 44225 awarded by the National Institutes of Health to James H. Morrissey.

This is a continuation of U.S. Serial No. 08/021615 filed February 19, 1993, entitled, "Treatment of Bleeding with Modified Tissue Factor In Combination with An Activator of FVII" which is a continuation-in-part of U.S. Serial No. 07/882,202 entitled "Treatment of Bleeding with Modified Tissue Factor in Combination with FVIIa" filed May 13, 1992, both by James H. Morrissey and Philip C. Comp.

Blood coagulation results from the production of thrombin, a proteolytic enzyme inducing platelet aggregation and cleaving fibrinogen to fibrin, which stabilizes the platelet plug. A number of proenzymes and procofactors circulating in the blood interact in this process through several stages during which they are sequentially or simultaneously converted to the activated form, ultimately resulting in the activation of prothrombin to thrombin by activated factor X (fXa) in the presence of factor Va, ionic calcium, and platelets.

Factor X can be activated by either of two pathways, termed the extrinsic and intrinsic pathways. The intrinsic pathway, or surface-mediated activation pathway, consists of a series of reactions where a protein precursor is cleaved to form an active protease, beginning

with activation of factor XII to factor XIIa, which converts factor XI to factor XIa, which, in the presence of calcium, converts factor IX to factor IXa. Factors IX and X can also be activated via the extrinsic pathway by tissue factor (TF) in combination with activated factor VII (factor VIIa; fVIIa). Activated factor IX, in the presence of calcium, phospholipid (platelets), and factor VIIIa, activates factor X to factor Xa.

Physiologically, the major pathway involved in coagulation is believed to be the extrinsic pathway, an essential step of which is tissue factor-mediated activation of factor VII to factor VIIa. Tissue factor is an integral membrane glycoprotein having a protein and a phospholipid component. It has been isolated from a variety of tissues and species and reported to have a molecular mass of between 42,000 and 53,000. DNA encoding tissue factor and methods for expression of the protein have now been reported, for example, in European Patent Application 0 278 776 by Genentech, Inc. and by J. H. Morrissey, et al. Cell 50, 129-135 (1987).

The complex of factor VIIa and its essential cofactor, TF, is the most potent known trigger of the clotting cascade. Factor VII is present in plasma at a concentration of 0.5 μg/ml plasma. In contrast, factor VIIa is present in plasma at trace levels of roughly 1 ng/ml. Accordingly, factor VII is normally in considerable excess over factor VIIa. Factor VIIa circulates with a relatively long half-life of about two hours in plasma. This is an unusual property among activated coagulation enzymes, which typically are inactivated very rapidly by various protease inhibitors in plasma.

Hemophilia A is characterized by the absence of active coagulation factor VIII or the presence of

inhibitors to factor VIII. Hemophilia B is characterized by the absence of active factor IX. Both types of hemophilia are associated with bleeding diatheses that can lead to crippling injuries or death. Traditionally, patients with either type of hemophilia were treated with infusion of plasma concentrates to stop bleeding episodes. The problems with the concentrates are many, especially infection, most recently with HIV. Highly purified factor VIII or factor IX have been introduced to obviate these problems, as have methods of treating factor concentrates to inactivate viruses. However, some patients develop high-titer, inhibitory antibodies to factor VIII. Therefore, such patients can no longer be treated with conventional factor VIII replacement therapy.

As described by Hedner and Kisiel, J. Clin. Invest. 71, 1836-1841 (1983), purified naturally produced factor VIIa can be administered to hemophilia A patients with high titers of antibodies against factor VIII:C and restore hemostasis in these patients. As reported by Brinkhous, et al., Proc. Natl. Acad. Sci. USA 86, 1382-1386 (1989), recombinant factor VIIa (rFVIIa) can be administered to hemophilic and von Willebrand disease dogs and stop bleeding in both hemophilic A and B dogs, but not the von Willebrand disease dogs. Telgt, et al., Thrombosis Res. 56, 603-609 (1989), reported that, at high levels, rFVIIa was believed to act by direct activation of factor X, in the presence of calcium and phospholipid but in the absence of TF. Teitel, Thrombosis and Haemostasis 66(5), 559-564 (1991), reported that the important ingredient in prothrombin complex concentrates for efficacy in treating hemophilia is factor VIIa.

Hedner, "Experiences with Recombinant Factor VIIa in Haemophiliacs" in Biotechnology of Plasma Proteins Lenfant, Mannucci, Sixma, eds., Curr. Stud. Hematol. Blood

Transf. No. 58, 63-68 (1991), review the use of prothrombin complex concentrates (effective in only 50 to 60% of the bleeds), as well as the use of plasma-derived (pFVIIa) and recombinant factor VIIa (rFVIIa). Dosages of 10 to 15 μg/kg of pFVIIa were effective in some hemophilia A patients. Safety studies in dogs and rabbits indicated that the recombinant factor VIIa was safe and efficacious at dosages of up to 150 μg/kg. A number of patients were also successfully treated, using dosages of between 54 μg/kg and 90 μg/kg during surgery and bleeding complications. Gringeri, et al., reported that treatment of hemophiliacs with rFVIIa is not always effective, even at dosages of 75 μg/kg at intervals of every two to three hours. The authors noted that perhaps larger dosages, more frequent infusions, and/or the concomitant use of antifibrinolytic medication might be required in such cases. rFVIIa is currently in clinical trials in the United States for treatment of hemophilia, particularly hemophilia in patients with inhibitors who do not benefit from conventional factor VIII or factor IX replacement therapy. Doses of rFVIIa currently employed are typically 45 to 90 μg rFVIIa/kg body weight, and are repeated every two to four hours. These doses are designed to achieve a level of circulating rFVIIa of approximately 4 μg/ml, extremely high compared to the normal plasma concentrations of FVII of approximately 0.5 μg/ml or FVIIa of approximately 1 ng/ml.

O'Brien, et al., J. Clin. Invest. 82, 206-211 (1988), reported that apo-TF, a delipidated preparation of the normally lipid-associated TF glycoprotein could be used to normalize bleeding in animals having antibodies to factor VIII. Since purified apo-TF is inactive unless incorporated into a phospholipid membrane, the theoretical basis for infusing apo-TF is the hypothesis that it would

spontaneously and preferentially incorporate into exposed membrane surfaces, particularly into damaged cells at the sites of injury. Subsequent studies have indicated there are dangers asscciated with the use of purified apo-TF in the treatment of hemophilia. The apo-TF can spontaneously incorporate into many types of lipid membranes and become active at sites where clotting is not desired, resulting in thrombosis or disseminated intravascularization (DIC). Indeed, O'Brien, et al., reported evidence of DIC in some animals receiving apo-TF, and Sakai and Kisiel, Thromb. Res. 60, 213-222 (1990), recently demonstrated that apo-TF will spontaneously combine with plasma lipoproteins to form active TF. This latter phenomenon is cause for concern because of a number of studies which have demonstrated that intravenous administration of active TF is a potent inducer of DIC.

Recently, a soluble, truncated form of TF (tTF) has been reported which retains some cofactor function towards factor VIIa as measured *in vitro* using purified proteins. However, this form of TF has been dismissed as an alternative to TF because it has almost no procoagulant activity when tested with normal plasma, as reported by Paborsky, et al., J. Biol. Chem. 266:21911-21916 (1991).

As described in U.S. Serial No. 07/683,682 entitled "Quantitative Clotting Assay for Activated Factor VII" filed April 10, 1991 by James H. Morrissey, the reason tTF was reported to lack procoagulant activity in the prior art is because, although tTF retains cofactor function toward factor VIIa, tTF had lost the ability to promote conversion of factor VII to factor VIIa. As a consequence, tTF can clot plasma only in conjunction with significantly elevated levels of factor VIIa, as compared to normal plasma, which contains only trace levels of factor VIIa.

It is therefore an object of the present invention to provide a method and composition for treatment of significant bleeding disorders, such as hemophilia, including those hemophiliacs with high titers of anti-factor VIII antibodies.

It is a further object of the present invention to provide a method and compositions for treatment of patients with bleeding disorders that are relatively safe and can be produced in commercial quantities.

Summary of the Invention

It has been discovered that it is possible to administer truncated tissue factor, the isolated extracellular domain of tissue factor, (tTF) in combination with factor VIIa (FVIIa), or truncated tissue factor in combination with an activator of factor VIIa such as the combination of factor Xa/phospholipid, factor IXa/phospholipid, thrombin, factor XIIa, or the factor VII activator from the venom of *Oxyuranus scutellatus*/ phospholipid, to treat bleeding disorders such as, for example, those associated with hemophilia or cirrhosis of the liver. The minimal effective dosages of both tTF and FVIIa are significantly and surprisingly less than the administration of either alone to stop bleeding.

Examples demonstrate safety and efficacy in normal and hemophilic dogs.

Brief Description of the Drawings

Figure 1a is a graph of dilute thromboplastin clotting time (seconds) for factor VIII-deficient plasma when tTF and FVIIa (ng/ml) are added (dark triangles) or when FVIIa alone (ng/ml) are added (dark circles).

Figure 1b is a graph of dilute thromboplastin clotting time (seconds) for normal plasma when tTF and

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Thus, in a first aspect, the present invention provides a composition for treatment of patients with prolonged or excessive bleeding, comprising (i) a soluble truncated tissue factor (tTF) comprised of the extracellular domain, or a functionally-equivalent fragment thereof, of wild-type tissue factor (TF), said truncated tissue factor characterised by its inability to bind to phospholipids and its inability to support conversion of factor VII to factor VIIa, and (ii) a substance selected from factor VIIa and activators of endogenous factor VII other than wild-type tissue factor, said substance effective to produce a plasma level of factor VIIa which in combination with said truncated tissue factor will control or stop said prolonged or excessive bleeding.

In a second aspect, the present invention provides a method of treating prolonged or excessive bleeding in a patient, said method comprising sequentially administering to said patient (i) a composition comprising a soluble truncated tissue factor (tTF) comprised of the extracellular domain, or a functionally-equivalent fragment thereof, of wild-type tissue factor (TF), said truncated tissue factor characterised by its inability to bind to phospholipids and its inability to support conversion of factor VII to factor VIIa, together with a pharmaceutically acceptable carrier, and (ii) a substance selected from factor VIIa and activators of endogenous factor VII other than wild-type tissue factor.

Brief Description of the Drawings

Figure 1a is a graph of dilute thromboplastin clotting time (seconds) for factor VIII-deficient plasma when tTF and FVIIa (ng/ml) are added (dark triangles) or when FVIIa alone (ng/ml) are added (dark circles).

Figure 1b is a graph of dilute thromboplastin clotting time (seconds) for normal plasma when tTF and

AUSTRALIAN PATENT OFFICE

FVIIa (ng/ml) are added (dark triangles) or when FVIIa alone (ng/ml) are added (dark circles).

Detailed Description of the Invention

Truncated tissue factor (tTF) is used as an adjuvant for factor VIIa (FVIIa) therapy of excessive bleeding in patients such as hemophiliacs. The tTF is administered in combination with the FVIIa, or a composition which activates endogenous FVII to form FVIIa, such as the combination of FXa and phospholipid, or immediately before or after the FVIIa. Whilst the invention is hereinafter particularly described in the form of a composition comprising tTF and FVIIa, it is to be understood that the reference to FVIIa may equally be regarded as a reference to an activator of FVII to FVIIa. The minimal effective dosage of FVIIa is significantly decreased by the tTF, while the thrombogenicity of the mixture is reduced as compared to a mixture of FVIIa and TF in its native form.

The advantage, and necessity, of using a combination of FVIIa plus tTF over tTF alone is that tTF is active in promoting blood clotting only in the presence of preformed FVIIa. The advantage of using the combination of FVIIa plus tTF over FVIIa alone is that FVIIa is approximately 22-fold more potent at clotting plasma in the presence of tTF than in the absence of tTF. Furthermore, as with FVIIa alone, the complex of tTF plus FVIIa activates factor X at markedly enhanced rates in the presence of negatively charged phospholipid such as phosphatidyl serine, even though tTF is not itself incorporated into lipid membranes. Normally, negatively charged phospholipids are sequestered into the inner leaflet of the plasma membrane of cells, and thus are not exposed to the cell surface unless the cells are damaged, or, in the case of platelets, the platelets have undergone surface activation. Thus, negatively charged phospholipids should be preferentially exposed on cell

AUSTRALIAN PATENT OFFICE

surfaces at anatomic sites that have experienced trauma sufficient to cause cell lysis and/or platelet activation. This means that the complex of tTF and FVIIa is predicted from *in vitro* studies to be active in promoting blood clotting preferentially at sites of injury, where it would be needed to confer hemostasis. Furthermore, tTF, by itself or in complex with FVIIa, remains a soluble entity and does not become incorporated into lipid membranes. This means that it should clear relatively rapidly from the circulation, diminishing the risk of thrombosis. Because of these properties, the complex of tTF and FVIIa should not cause systemic activation of the blood clotting system leading to DIC, as can injection of wild-type TF. These *in vitro* results are consistent with results from *in vivo* studies in normal and hemophiliac dogs described below.

The combination of tTF plus FVIIa should be useful for treatment of hemophilia A, hemophilia B, congenital or acquired deficiencies in any other blood coagulation factor, or platelet defects. Other patients that can be treated with the combination include patients suffering from severe trauma, postoperative bleeding or those with cirrhosis.

Truncated Tissue Factor

As used herein, "truncated tissue factor" is a soluble tissue factor having only the extracellular domains, which is not bound to a phospholipid membrane surface, and therefore does not support conversion of fVII to fVIIa, as described in U.S. Serial No. 07/683,682 filed April 10, 1991, corresponding to International Application PCT/US92/02898 filed April 9, 1992, the teachings of which are incorporated herein. In the preferred embodiment, truncated tissue factor is a recombinant protein produced *in vitro* in cell culture using a mammalian cell line such

as CHO-K1 cells, American Type Culture Collection CCL 61. These cells are stably transfected with a mutant form of the human tissue factor cDNA carried in a commercially available expression vector, and secrete a form of tissue factor consisting only of amino acids 1-219 (numbered according to Morrissey, et al., Cell 50:129-135 (1987), the teachings of which are incorporated herein).

The recombinant, truncated tissue factor is purified from the culture medium using an immobilized monoclonal antibody to human tissue factor, such as TF9-5B7, described in Morrissey, et al. Thromb. Res. 52:247-261 (1988). Hybridomas for production of monoclonal antibodies can be propagated by ascites growth and the monoclonal antibodies (MAbs) purified from ascites fluid using the BioRad MAPS II system for mAb purification, as described by Morrissey, et al., Thromb. Res. 52:247-261 (1988).

The TF9-5B7 is coupled to Affigel™ beads. Detergent is not used during the purification of TF_{219}. After removal of cellular debris by centrifugation, the culture medium is made 25 mM in Tris.HCl (pH 7.4), 10 mM in sodium EDTA (pH 7.4), and 0.1% in sodium azide by the addition of concentrated stock solutions. In order to remove proteins that bind to agarose beads non-specifically, the culture medium is gently agitated for 4 hr at 4°C with AffiGel™-10 beads that had previously been blocked chemically with glycine ethyl ester (GEE-AffiGel). The GEE-AffiGel beads are removed by filtration through a sintered glass funnel, and the supernatant is agitated overnight at 4°C with the MAb TF9-5B7 coupled to AffiGel beads (typically 2 ml of beads). The TF9-5B7-AffiGel beads are collected on a sintered glass funnel, and the beads are washed on the funnel with 100 ml of TBS-EDTA (TBS = 100 mM NaCl, 50 mM Tris.HCl pH 7.4, 0.02% sodium azide; TBS-EDTA = TBS with

10 mM EDTA included). The beads are then transferred to a chromatography column and washed with 40 ml TBS followed by 40 ml of a solution consisting of 1 M NaCl, 10 mM Tris.HCl pH 7.4, 0.02% sodium azide. Truncated TF is eluted from the beads using 100 mM glycine.HCl pH 2.5, with 1 ml fractions being collected into tubes containing 57 µl 1 M Tris base (to immediately neutralize the acidic glycine buffer). Fractions containing protein are detected using the BCA protein assay (Pierce), pooled, dialyzed against TBS, and then stored at -70°C. Protein concentrations are determined using standard methods such as the BCA assay (Pierce Chemical Co.) based on a bovine serum albumin standard of known concentration.

For production of recombinant truncated TF, cells are typically grown to confluence in roller bottles in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% iron-supplemented calf serum (HyClone Laboratories, Logan, Utah). Upon reaching confluence, the cultures are shifted to DMEM containing 2% serum, and culture medium is collected every four days thereafter.

The nucleotide and amino acid sequence of truncated tissue factor (tTF) is shown below as SEQ. ID. NO: 1 and 2, respectively. The truncated tissue factor protein lacks the predicted transmembrane and cytoplasmic domains of tissue factor. This version of the protein retains cofactor activity, as reported by Ruf, et al., Thromb. Haemost. 62, 347 (abstract) (1989) and Ruf, et al., J. Biol. Chem. 266: 2158-2166 (1991). This truncated form of tissue factor fails to support conversion of factor VII to VIIa, allowing it to be used in a specific clotting assay for factor VIIa free from interference by factor VII.

Since the vast majority of FVII in plasma is in the inactive, zymogen form, and since tTF is selectively deficient in promoting conversion of factor VII to VIIa,

tTF has extremely little procoagulant activity towards normal plasma. Since tTF lacks the membrane-anchoring domain of the protein, it lacks the ability to become incorporated into exposed phospholipid surfaces as wild-type apo-TF can. Although the deficiency of tTF in the conversion of FVII to FVIIa was not previously appreciated, its extremely low procoagulant activity when tested with normal plasma has made it appear to be unacceptable as a therapeutic agent to control bleeding in hemophilia.

The essential difference between truncated tissue factor and wild-type tissue factor is that truncated tissue factor is no longer tethered to the phospholipid membrane surface. It is therefore expected that other methods for preparing truncated tissue factor can be used to generate an essentially equivalent soluble form of tissue factor that retains FVIIa cofactor activity while no longer stimulating conversion of factor VII to factor VIIa. Methods include chemical and/or enzymatic cleavage of wild-type tissue factor to separate the predicted extracellular domain from the transmembrane region. Recombinant human TF is available from Calbiochem Corporation. Precise positioning of the stop codon following amino acid 219 is believed to not be essential to make functional truncated TF, and other placements of a stop codon near amino acid 219 are predicted to yield an essentially equivalent product with respect to its ability in conjugation with FVIIa to serve as a treatment for bleeding disorders.

Activated Factor VII

Factor VII can be prepared as described by Fair, Blood 62, 784-791 (1983). The coding portion of the human factor VII cDNA sequence reported by Hagen et al., Proc. Natl. Acad. Sci. USA 83:2412-2416 (1986) is shown below as

SEQ. ID. NO: 3, along with the translated amino acid sequence, SEQ. ID. NO: 4. The amino acid sequence from 1 to 60 corresponds to the pre-pro/leader sequence that is removed by the cell prior to secretion. The mature FVII polypeptide chain consists of amino acids 61 to 466. FVII is converted to FVIIa by cleavage of a single peptide bond between arginine-212 and isoleucine-213.

FVII can be converted *in vitro* to fVIIa by incubation of the purified protein with factor Xa immobilized on Affi-Gel™ 15 beads (Bio-Rad). Conversion can be monitored by SDS-polyacrylamide gel electrophoresis of reduced samples. Free factor Xa in the fVIIa preparation can be detected with the chromogenic substrate methoxycarbonyl-D-cyclohexylglycyl-glycyl-arginine-*p*-nitroanilide acetate (Spectrozyme™ FXa, American Diagnostica, Greenwich, CT) at 0.2 mM final concentration in the presence of 50 mM EDTA.

Recombinant fVIIa can also be purchased from Novo Biolabs (Danbury, CT).

Formation of FVIIa *in vivo*

Alternatively, the FVIIa can be formed *in vivo*, at the time of, or shortly before, administration of the truncated tissue factor. In a preferred embodiment, endogenous FVII is converted into FVIIa by infusion of an activator of VIIa, such as factor Xa (FXa) in combination with phospholipid (PCPS).

Activators of factor VII *in vivo* include FXa/PCPS, FIXa/PCPS, thrombin, FXIIa, and the FVII activator from the venom of *Oxyuranus scutellatus* in combination with PCPS. These have been shown to activate FVII to FVIIa <u>in vitro</u>, although never in combination with truncated tissue factor:

FXa and Thrombin: Radcliffe, R. and Nemerson, Y. (1975). Activation and control of factor VII by activated

factor X and thrombin: Isolation and characterization of a single chain form of factor VII. J. Biol. Chem. 250:388-395. This paper points out that activation of FVII by FXa is accelerated when phospholipids are present.

FIXa and FXa: Masys, D.R., Bjaj, S.P. and Rapaport, S.I. (1982). Activation of human factor VII by activated factors IX and X. Blood 60:1143-1150. This paper describes activation of factor VII by both factors IXa and Xa, and the fact that phospholipids accelerate both reactions.

FXa plus phospholipids in vivo: Giles, A.R., Mann, K.G. and Nesheim, M.E. (1988). A combination of factor Xa and phosphatidylcholine-phosphatidylserine vesicles bypasses factor VIII in vivo. Br. J. Haematol. 69:491-497. This paper describes the factor VIII bypassing activity of FXa/PCPS in hemophilic dogs. It does not deal with activation of factor VII.

FXIIa: Kisiel, W., Fujikawa, K. and Davie, E.W. (1977). Activation of bovine factor VII (proconvertin) by factor XIIa (activated Hageman factor). Biochemistry 16:4189-4194. Describes activation of bovine FVII by FXIIa (no phospholipids).

Snake venom activator of FVII: Nakagaki, T., Lin, P. and Kisiel, W. (1992). Activation of human factor VII by the prothrombin activator from the venom of Oxyuranus scutellatus (Taipan snake). Thromb. Res. 65:105-116. This paper describes the isolation of an enzyme from this snake venom which, in combination with phospholipids, will activate factor VII to VIIa in vitro.

Activation of FVII to FVIIa for Xa/PCPS in vivo has also been measured directly.

In general, the factor VII activator is administered in a dosage between 1 and 10 μg/ml of carrier.

The phospholipid can be provided in a number of forms but the preferred form is as phosphatidyl choline/phosphatidyl serine vesicles (PCPS). The PCPS vesicle preparations and the method of administration of Xa/PCPS is described in Giles, et al., (1988), the teachings of which are specifically incorporated herein. Other phospholipid preparations can be substituted for PCPS, so long as they accelerate the activation of FVII by FXa. Effectiveness, and therefore determination of optimal composition and dose, can be monitored as described below.

A highly effective dose of Xa/PCPS, which elevates FVIIa levels in vivo in the chimpanzee, was 26 pmoles FXa + 40 pmoles PCPS per kg body weight. That dose yielded an eighteen fold increase in endogenous levels of FVIIa (to 146 ng/ml). A marginally detectable effect was observed using a smaller dose in dogs, where the infusion of 12 pmoles FXa + 19 pmoles PCPS per kg body weight yielded a three fold increase in endogenous FVIIa levels. Accordingly, doses of FXa that are at least 12 pmoles FXa per kg body weight, and preferably 26 pmoles FXa per kg body weight, should be useful. Doses of PCPS that are at least 19 pmoles PCPS per kg body weight, and preferably 40 pmoles PCPS per kg body weight, are similarly useful.

The effectiveness of any infusible FVII activator can be monitored, following intravenous administration, by drawing citrated blood samples at varying times (at 2, 5, 10, 20, 30, 60, 90 and 120 min) following a bolus infusion of the activator, and preparing platelet-poor plasma from the blood samples. The amount of endogenous FVIIa can then be measured in the citrated plasma samples by performing our tTF-based FVIIa clotting assay. Desired levels of endogenous FVIIa would be the same as the target levels of plasma FVIIa indicated for co-infusion of

purified FVIIa and tTF. Therefore, other activators of FVII could be tested in vivo for generation of FVIIa, without undue experimentation, and the dose adjusted to generate the desirable levels of FVIIa, using the tTF-based FVIIa assay of plasma samples. The proper dose of the FVII activator (yielding the desired level of endogenous FVIIa) can then be used in combination with the recommended amounts of tTF.

Doses can be timed to provide prolong elevation in FVIIa levels. Preferably doses would be administered every two hours until the desired hemostatic effect is achieved, and then repeated as needed to control bleeding. The half-life of FVIIa in vivo has been reported to be approximately two hours, although this could vary with different therapeutic modalities and individual patients. Therefore, the half-life of FVIIa in the plasma in a given treatment modality should be determined with the tTF-based clotting assay.

Administration of the combination of the tTF and FVIIa

The tTF and VIIa are administered by infusion in the preferred embodiment, using a pharmaceutically acceptable carrier such as saline or buffered saline. The tTF and VIIa can also be administered topically either by direct application using a conventional topical base such as petrolatum or a water based gel, or as an aerosol spray.

The tTF is administered in a dosage effective to produce in the plasma an effective level of between 100 ng/ml and 50 μg/ml, or a preferred level of between 1 μg/ml and 10 μg/ml or 60 to 600 μg/kg body weight, when administered systemically; or an effective level of between 10 μg/ml and 50 μg/ml, or a preferred level of between 10 μg/ml and 50 μg/ml, when administered topically.

The FVIIa is administered in a dosage effective to produce in the plasma an effective level of between 20 ng/ml and 10 μg/ml (1.2 to 600 μg/kg), or a preferred level of between 40 ng/ml and 4 μg/ml (2.4 to 240 μg/kg), or a level of between 1 to 10 μg FVIIa/ml when administered topically.

The FVIIa activator is administered in a dosage effective to produce in the plasma an effective level of FVIIa, as defined above. However, the maximum amount of FVIIa converted from endogenous FVII is about 700 ng per ml.

In general, one would administer tTF and FVIIa to produce levels of up to 10 μg tTF/ml plasma and between 40 ng and 4 μg FVIIa/ml plasma. For hemophilic patients, one would administer tTF and FVIIa to produce levels of up to 10 μg tTF and between 100 and 300 ng FVIIa/ml. For patients with cirrhosis, one would administer the same amount of tTF but up to 1 μg FVIIa/ml plasma.

A preferred composition according to the present invention comprises tTF in a concentration of between 100 ng and 50 μg/ml of a carrier and an activator of FVII in a concentration of between 1 and 10 μg/ml of a carrier. Another preferred composition according to the present invention is suitable for topical administration and comprises tTF in a concentration of between 100 ng and 50 μg/ml of a carrier and factor VIIa in a concentration of between 1 and 10 μg/ml of carrier.

Since tTF cannot stimulate conversion of FVII to FVIIa, it should only be functional in conjunction with elevated factor VIIa levels. Therefore, tTF is expected to be an effective hemostatic agent only in association with FVIIa therapy or in individuals who have elevated FVIIa levels for some other reason.

Other conditions that can be treated with this combination include surgical bleeding from the microvasculature, bleeding at skin graft harvest sites,

AUSTRALIAN PATENT OFFICE

postoperative bleeding, including following orthopedic surgery, brain surgery or brain trauma, bleeding secondary to thrombocytopenia, and platelet dyfunction.

Example 1: Safety of tTF and FVIIa in normal dogs.

Three beagle dogs were treated with either tTF or tTF plus FVIIa.

AUSTRALIAN PATENT OFFICE

Dog #5533 was treated with tTF alone, in a dosage of 60 μg tTF/kg body weight by intravenous bolus infusion. ELISA studies showed 1.23 μg tTF/ml in plasma at time zero. Half-life of tTF was 2.2 hr in this dog.

Dog #5534 was also treated with tTF alone, in a dosage of 41 μg tTf/kg body weight by intravenous bolus infusion. Assuming a blood volume of 60 ml/kg, this should have produced a level of 0.67 μg tTF/ml in the blood. ELISA studies showed 0.60 μg tTF/ml in plasma at time zero. Half-life of tTF was 1.3 hr in this dog.

Dog #5734 was treated with tTF in combination with FVIIa, in a dosage of 41 μg tTf/kg body weight and 6.37 μg Novo recombinant factor VIIa/kg body weight by intravenous bolus infusion. ELISA studies showed 0.51 μg tTF/ml in plasma at time zero. Half-life of tTF was biphasic (1 hr followed by 3.0 hr) in this dog.

The dogs all remained healthy and active. Blood tests of the clotting system indicated no significant decrease in fibrinogen levels or platelet counts and no measurable increase in fibrin degradation products. Prothrombin times and APTT times were normal, as were white blood cell counts, and red blood cell counts. Therefore, administration of either tTF alone, or tTF in conjunction with FVIIa, caused no measurable disseminated intravascular coagulation or other detectable coagulopathy.

A complete necropsy was performed on the animal receiving truncated tissue factor and factor VIIa. No evidence of thrombosis was found in veins, arteries or in the capillaries. There was no evidence on gross and microscopic examination of myocardial infarction or of cerebral infarction (stroke).

Example 2: ***In vitro*** **correction of clotting time of hemophilic plasma with tTF in combination with FVIIa.**

A modified prothrombin test (PT) was performed with diluted thromboplastin (since hemophiliacs have normal PT's unless the thromboplastin is diluted) using either normal or congenital factor VIII deficient (Hemophilia A) plasma. Sigma Chemical Co. rabbit brain thromboplastin was diluted 1:500 with TBS/0.1% BSA/rabbit brain cephalin (Sigma Chemical Co.).

12 x 75 mm glass test tubes were pre-warmed in a 37°C water bath.

Diluted thromboplastin was added (0.1 ml) and allowed to warm to 37°C for more than two minutes.

Plasma sample (0.1 ml) was added and allowed to warm to exactly 30 sec.

Pre-warmed 25 mM $CaCl_2$ (at 37°C) was added and the clotting time was determined by the manual tilt-tube method.

The results are shown in Figure 1a and b. Figure 1a is a graph of dilute thromboplastin clotting time (seconds) for factor VIII-deficient plasma when tTF (1 μg/ml) and varying concentrations of FVIIa (ng/ml) are added (dark triangles) or when varying concentrations of FVIIa alone (ng/ml) are added (dark circles). Figure 1b is a graph of dilute thromboplastin clotting time (seconds) for normal plasma when tTF (1 μg/ml) and varying concentrations FVIIa (ng/ml) are added (dark triangles) or when varying concentration of FVIIa alone (ng/ml) are added (dark circles). The clotting time of the hemophilic plasma without any added FVIIa or tTF was 88.5 sec which is indicated by the upper dotted horizontal line. The clotting time of normal plasma without added FVIIa or tTF was 53.0 sec which is indicated by the lower dotted

horizontal line. Without added tTF, the 88.5 sec clotting time of the hemophilic plasma was reduced to that of normal plasma (53 sec) at 808 ng FVIIa/ml of plasma. With added tTF (at 1 μg/ml), the clotting time of the hemophilic plasma was reduced to that of normal plasma at 36.8 ng FVIIa/ml plasma.

Therefore, in the presence of 1 μg/ml tTF in plasma, correction of the prolonged clotting time of hemophilic plasma was achieved at a level of added FVIIa that was 22-fold lower than in the absence of added tTF. In addition, tTF was not able to correct the prolonged clotting time of hemophilic plasma in the absence of added FVIIa.

Example 3: Efficacy of the combination of tTF and FVIIa in the treatment of Hemophilic Dogs.

As described by Brinkhous, e al., Proc. Natl. Acad. Sci. USA 82, 8752-8756 (1985), Graham, et al., J. Exp. Med. 90, 97-111 (1949), and Brinkhous, et al., Ann. N.Y. Acad. Sci. 370, 191-204 (1981), a colony of hemophilic dogs has been developed at the University of North Carolina at Chapel Hill. Dogs have hemophilia A. A test (secondary cuticle bleeding time) is used to measure bleeding tendency.

A modified toenail bleeding time (BT) is used to test the hemostatic effectiveness of infused preparations. The paw of the front leg is warmed by placing it in isotonic saline at 37°C and a toenail is trimmed to expose only the distal matrix. The bleeding nail is placed in isotonic saline at 37°C and the time until cessation of bleeding recorded as the primary BT. At 2 to 4 hours, the site is shaved to remove the clot and as little nail matrix as possible. The paw is again placed in saline at 37°C. A discrete stream of extruding blood is visible. With hemophilic dogs in the absence of treatment, bleeding typically continues for 30 min or longer. This time to

cessation of bleeding is recorded as a secondary BT. For normal dogs, the primary BT is two to five minutes and the secondary BT is less than five minutes. For hemophilic dogs the primary BT is similar to that of normal dogs while secondary BT is greater than 15 minutes.

Dog #V02 was administered a low dose of FVIIa, 6 μg FVIIa/kg body weight, which was designed to provide only a weak hemostatic effect. After 15 min equilibrium, secondary cuticle bleeding time was measured at 2 min 30 sec, which spontaneously rebled for 5 min 15 sec. Rebleeding challenge (wipe off clot with gauze): 9 min 20 sec bleeding time, which again spontaneously rebled for 15 min, at which time the nail was cauterized to prevent further bleeding. It was difficult to stop bleeding and oozing from the phlebotomy sites in this dog, which continued after FVIIa administration.

The results indicate that this dosage of FVIIa had a very weak hemostatic effect.

The dog was then administered 42 μg tTF/kg body weight. After 15 min equilibration, secondary cuticle bleeding time was measured at 50 sec, which did not spontaneously rebleed. This was dramatically shorter than when the animal had received FVIIa alone. Wiping the clot off with gauze to provoke rebleeding caused rebleeding for only 35 sec, and no subsequent rebleeding was observed. Oozing from two phlebotomy sites in forepaw veins also stopped following infusion of tTF.

The results indicated that tTF in combination with low dose FVIIa has an excellent hemostatic effect.

The same dog was administered 42 μg tTF/kg body weight, without FVIIa, two days later (since the half-lives of both tTF and FVIIa are about 2 hr, this was more than sufficient to ensure clearance of either substance from the plasma). After 15 minutes equilibration, the

secondary cuticle bleeding time was greater than 15 minutes and had to be stopped by cauterization.

The results indicated that tTF alone had no measurable hemostatic effect.

The dog was then administered 6 μg FVIIa/kg body weight. After 15 minutes equilibration, secondary cuticle bleeding time was measured at 3 minutes 15 seconds, which did not spontaneously rebleed. Wiping the clot off with gauze caused rebleeding for 3 minutes, and no subsequent rebleeding was observed.

The results again demonstrate that tTF in combination with low dose FVIIa has a very good hemostatic effect.

Example 4: Infusion of Factor Xa in combination with phospholipid vesicles (PCPS) to generate Factor VIIa to inhibit bleeding.

Three chimpanzees were infused with a bolus injection of FXa/PCPS as described by Giles, et al., (1988), the teachings of which are incorporated herein by reference. Briefly, factor Xa/PCPS was infused intravenously. The chimpanzee receiving the highest dose received 26 pmoles FXa + 40 pmoles PCPS per kg body weight. Plasma samples were drawn at the following times after infusion, giving the indicated plasma FVIIa levels:

	Plasma FVIIa (ng/ml)
Pre-infusion	8.0
2 min post-infusion	24.6
5 " " "	65.4
10 " " "	125.5
15 " " "	146.5
20 " " "	132.0
20 " " "	101.8
60 " " "	43.6
90 " " "	4.4

The results indicate that infusion of factor Xa with PCPS greatly increased factor VIIa levels.

Modifications and variations of the present invention, a method and compositions for the treatment of excessive bleeding, will be obvious from the foregoing detailed description and are intended to come within the scope of the appended claims.

AUSTRALIAN PATENT OFFICE

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Morrissey, James H.
Comp, Philip C.

(ii) TITLE OF INVENTION: Truncated Tissue Factor and FVIIa or FVII Activator for Blood Coagulation

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: Dallas
(D) STATE: Texas
(E) COUNTRY: US
(F) ZIP: 75270-2197

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/882202
(B) FILING DATE: 13-MAY-1992

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/021615
(B) FILING DATE: 19-FEB-1993

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Trujillo, Doreen Y.
(B) REGISTRATION NUMBER: 35,719
(C) REFERENCE/DOCKET NUMBER: OMRF B34290CIPC/PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 214-939-4500
(B) TELEFAX: 214-939-4600

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 795 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(F) TISSUE TYPE: Fibroblast

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 34..786
(D) OTHER INFORMATION: /product= "Truncated Tissue Factor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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     25                  30                  35

GCA GCA TAT AAT TTA ACT TGG AAA TCA ACT AAT TTC AAG ACA ATT TTG       198
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             75                  80                  85

GAG TGT GAC CTC ACC GAC GAG ATT GTG AAG GAT GTG AAG CAG ACG TAC       342
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         90                  95                 100

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120                 125                 130                 135

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AGG AAC AAC ACT TTC CTA AGC CTC CGG GAT GTT TTT GGC AAG GAC TTA       582
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    185                 190                 195

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Ala Lys Thr Asn Thr Asn Glu Phe Leu Ile Asp Val Asp Lys Gly Glu
200                 205                 210                 215

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Asn Tyr Cys Phe Ser Val Gln Ala Val Ile Pro Ser Arg Thr Val Asn
                220                 225                 230

CGG AAG AGT ACA GAC AGC CCG GTA GAG TGT ATG GGC CAG GAG AAA GGG        774
Arg Lys Ser Thr Asp Ser Pro Val Glu Cys Met Gly Gln Glu Lys Gly
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 251 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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         35                  40                  45

Thr Asn Phe Lys Thr Ile Leu Glu Trp Glu Pro Lys Pro Val Asn Gln
     50                  55                  60

Val Tyr Thr Val Gln Ile Ser Thr Lys Ser Gly Asp Trp Lys Ser Lys
 65                  70                  75                  80

Cys Phe Tyr Thr Thr Asp Thr Glu Cys Asp Leu Thr Asp Glu Ile Val
                 85                  90                  95

Lys Asp Val Lys Gln Thr Tyr Leu Ala Arg Val Phe Ser Tyr Pro Ala
            100                 105                 110

Gly Asn Val Glu Ser Thr Gly Ser Ala Gly Glu Pro Leu Tyr Glu Asn
        115                 120                 125

Ser Pro Glu Phe Thr Pro Tyr Leu Glu Thr Asn Leu Gly Gln Pro Thr
    130                 135                 140

Ile Gln Ser Phe Glu Gln Val Gly Thr Lys Val Asn Val Thr Val Glu
145                 150                 155                 160
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                165                 170                 175

Asp Val Phe Gly Lys Asp Leu Ile Tyr Thr Leu Tyr Tyr Trp Lys Ser
            180                 185                 190

Ser Ser Ser Gly Lys Lys Thr Ala Lys Thr Asn Thr Asn Glu Phe Leu
        195                 200                 205

Ile Asp Val Asp Lys Gly Glu Asn Tyr Cys Phe Ser Val Gln Ala Val
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1440 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(F) TISSUE TYPE: Blood

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 36..1433
(D) OTHER INFORMATION: /product= "Tissue Factor"
/note= "Coding portion of human factor VIII cDNA"
/citation= ([1])

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GGG GTC GCT AAG GCC TCA GGA GGA GAA ACA CGG GAC ATG CCG TGG AAG        149
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CCG GGG CCT CAC AGA GTC TTC GTA ACC CAG GAG GAA GCC CAC GGC GTC        197
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                 75                  80                  85

GCC CGG GAG ATC TTC AAG GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT        341
Ala Arg Glu Ile Phe Lys Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile
             90                  95                 100

TCT TAC AGT GAT GGG GAC CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG        389
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        105                 110                 115

GGC TCC TGC AAG GAC CAG CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT        437
Gly Ser Cys Lys Asp Gln Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro
    120                 125                 130

GCC TTC GAG GGC CGG AAC TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC        485
Ala Phe Glu Gly Arg Asn Cys Glu Thr His Lys Asp Asp Gln Leu Ile
135                 140                 145                 150

TGT GTG AAC GAG AAC GGC GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG        533
Cys Val Asn Glu Asn Gly Gly Cys Glu Gln Tyr Cys Ser Asp His Thr
                155                 160                 165

GGC ACC AAG CGC TCC TGT CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA        581
Gly Thr Lys Arg Ser Cys Arg Cys His Glu Gly Tyr Ser Leu Leu Ala
            170                 175                 180

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215                 220                 225                 230

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TGG GTG GTC TCC GCG GCC CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG        821
Trp Val Val Ser Ala Ala His Cys Phe Asp Lys Ile Lys Asn Trp Arg
            250                 255                 260

AAC CTG ATC GCG GTG CTG GGC GAG CAC GAC CTC AGC GAG CAC GAC GGG        869
Asn Leu Ile Ala Val Leu Gly Glu His Asp Leu Ser Glu His Asp Gly
        265                 270                 275

GAT GAG CAG AGC CGG CGG GTG GCG CAG GTC ATC ATC CCC AGC ACG TAC        917
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Pro Val Val Leu Thr Asp His Val Val Pro Leu Cys Leu Pro Glu Arg
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GGC TGG GGC CAG CTG CTG GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG       1109
Gly Trp Gly Gln Leu Leu Asp Arg Gly Ala Thr Ala Leu Glu Leu Met
        345                 350                 355

GTG CTC AAC GTG CCC CGG CTG ATG ACC CAG GAC TGC CTG CAG CAG TCA       1157
Val Leu Asn Val Pro Arg Leu Met Thr Gln Asp Cys Leu Gln Gln Ser
    360                 365                 370

CGG AAG GTG GGA GAC TCC CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC       1205
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375                 380                 385                 390

GGC TAC TCG GAT GGC AGC AAG GAC TCC TGC AAG GGG GAC AGT GGA GGC       1253
Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly
                395                 400                 405

CCA CAT GCC ACC CAC TAC CGG GGC ACG TGG TAC CTG ACG GGC ATC GTC       1301
Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val
            410                 415                 420

AGC TGG GGC CAG GGC TGC GCA ACC GTG GGC CAC TTT GGG GTG TAC ACC       1349
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        425                 430                 435

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    440                 445                 450

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 466 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Gly Cys Leu Ala Ala Gly Gly Val Ala Lys Ala Ser Gly Gly Glu Thr
            20                  25                  30
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Arg Asp Met Pro Trp Lys Pro Gly Pro His Arg Val Phe Val Thr Gln
          35                  40                  45

Glu Glu Ala His Gly Val Leu His Arg Arg Arg Arg Ala Asn Ala Phe
      50                  55                  60

Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu Cys Lys Glu Glu
 65                  70                  75                  80

Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys Asp Ala Glu Arg
                 85                  90                  95

Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp Gln Cys Ala Ser
            100                 105                 110

Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln Leu Gln Ser Tyr
        115                 120                 125

Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn Cys Glu Thr His
    130                 135                 140

Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly Gly Cys Glu Gln
145                 150                 155                 160

Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys Arg Cys His Glu
                165                 170                 175

Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr Pro Thr Val Glu
            180                 185                 190

Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg Asn Ala Ser Lys
        195                 200                 205

Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro Lys Gly Glu Cys
    210                 215                 220

Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln Leu Cys Gly Gly
225                 230                 235                 240

Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala His Cys Phe Asp
                245                 250                 255

Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu Gly Glu His Asp
            260                 265                 270

Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg Val Ala Gln Val
        275                 280                 285

Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn His Asp Ile Ala
    290                 295                 300

Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp His Val Val Pro
305                 310                 315                 320

Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr Leu Ala Phe Val
                325                 330                 335

Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu Asp Arg Gly Ala
            340                 345                 350
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Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg Leu Met Thr Gln
        355                 360                 365

Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser Pro Asn Ile Thr
    370                 375                 380

Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys
385                 390                 395                 400

Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp
                405                 410                 415

Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly
            420                 425                 430

His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln
        435                 440                 445

Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro
    450                 455                 460

Phe Pro
465
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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. A composition for treatment of patients with prolonged or excessive bleeding, comprising (i) a soluble truncated tissue factor (tTF) comprised of the extracellular domain, or a functionally-equivalent fragment thereof, of wild-type tissue factor (TF), said truncated tissue factor characterised by its inability to bind to phospholipids and its inability to support conversion of factor VII to factor VIIa, and (ii) a substance selected from factor VIIa and activators of endogenous factor VII other than wild-type tissue factor, said substance effective to produce a plasma level of factor VIIa which in combination with said truncated tissue factor will control or stop said prolonged or excessive bleeding.

2. A composition according to claim 1, wherein said substance effective to produce a plasma level of factor VIIa is factor VIIa.

3. A composition according to claim 1, wherein said substance effective to produce a plasma level of factor VIIa is an activator of endogenous factor VII.

4. The composition of any of claims 1 to 3 wherein said truncated tissue factor is in a dosage for administration to a patient in an amount effective to produce a concentration of between 100 ng and 50 µg truncated tissue factor per millilitre of plasma and said substance is in a dosage in an amount effective to produce levels of between 20 ng and 700 µg factor VIIa per millilitre of plasma.

5. The composition according to claim 2, wherein the truncated tissue factor and factor VIIa are in a pharmaceutically acceptable carrier for topical administration to a patient, and the truncated tissue

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factor is in a concentration of between 100 ng and 50 µg/ml of carrier and the factor VIIa is in a concentration of between 1 and 10 µg/ml of carrier.

6. The composition according to claim 5, wherein said combination is formulated so that a dose of said combination provides a plasma level of between about 100 ng and 50 µg truncated tissue factor per millilitre of plasma and between about 20 ng and 10 µg factor VIIa per millilitre of plasma.

7. The composition of claim 5, wherein said truncated tissue factor is in a concentration of between 10 and 50 µg/ml.

8. The composition of claim 3, further comprising a pharmaceutically acceptable carrier and wherein said truncated tissue factor is in a concentration of between 100 ng and 50 µg/ml of carrier and wherein the activator is in a concentration of between 1 and 10 µg/ml of carrier.

9. The composition of claim 3 wherein said activator of endogenous factor VII is selected from the group consisting of factor Xa in combination with phospholipid, factor IXa in combination with phospholipid, thrombin, factor XIIa, and the FVII activator from the venom of *Oxyuranus scutellatus* in combination with phospholipid.

10. A composition according to any one of the preceding claims wherein said truncated tissue factor has the amino acid sequence of 1-219 of SEQ ID NO:2.

11. A method of treating prolonged or excessive bleeding in a patient comprising administering to said patient a pharmaceutically effective amount of a composition according to any one of the preceding claims.

AUSTRALIAN PATENT OFFICE

12. A method according to claim 11, wherein said patient suffers from hemophilia A or hemophilia B.

13. A method according to claim 11, wherein said patient is suffering from severe trauma, postoperative bleeding or cirrhosis.

14. A method of treating prolonged or excessive bleeding in a patient said method comprising sequentially administering to said patient (i) a composition comprising a soluble truncated tissue factor (tTF) comprised of the extracellular domain, or a functionally-equivalent fragment thereof, of wild-type tissue factor (TF), said truncated tissue factor characterised by its inability to bind to phospholipids and its inability to support conversion of factor VII to factor VIIa, together with a pharmaceutically acceptable carrier, and (ii) a substance selected from factor VIIa and activators of endogenous factor VII other than wild-type tissue factor.

15. A method according to claim 14, wherein said substance is an activator of endogenous factor VII is selected from the group consisting of factor Xa in combination with phospholipid, factor IXa in combination with phospholipid, thrombin, factor XIIa, and the FVII activator from the venom of *Oxyuranus scutellatus* in combination with phospholipid, and is administered prior to said composition comprising tTF.

16. A method according to claim 14 or 15, wherein said patient suffers from hemophilia A or hemophilia B.

17. A method according to claim 14 or 15, wherein said patient is suffering from severe trauma, postoperative bleeding or cirrhosis.

DATED this 18th day of December 1996.

OKLAHOMA MEDICAL RESEARCH FOUNDATION
Patent Attorneys for the Applicant:

F.B. RICE & CO.

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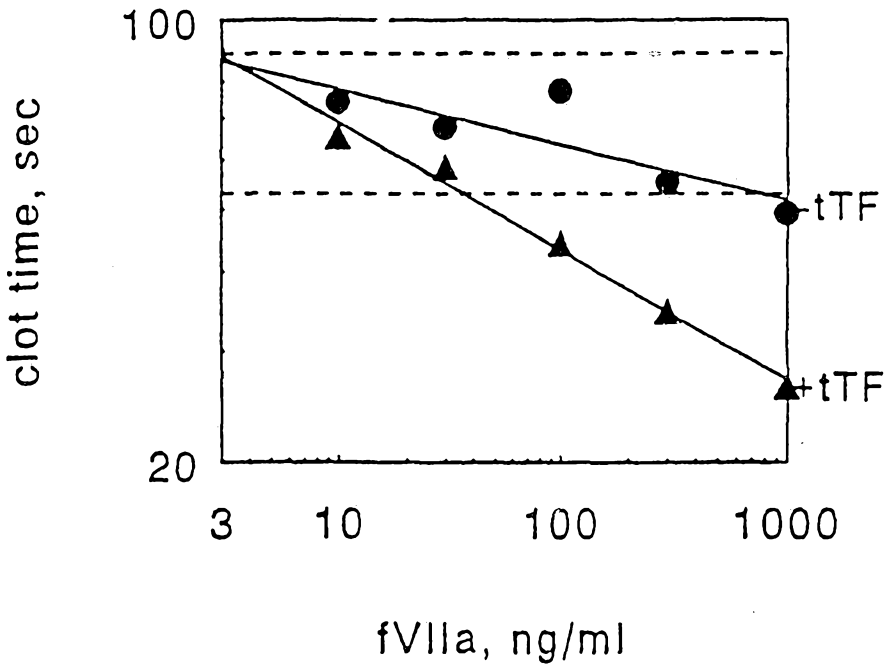

FIG. 1A

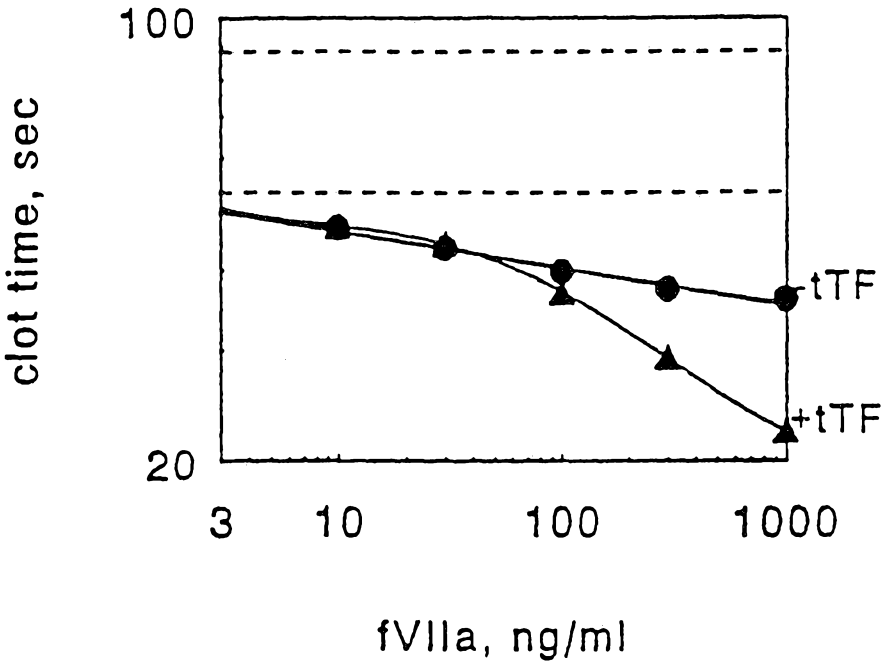

FIG. 1B

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/04493

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K37/547; //(A61K37/547,37:02)

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
A	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 266, no. 4, 5 February 1991, BALTIMORE US pages 2158 - 2166 W. RUF ET AL. 'PHOSPHOLIPID-INDEPENDENT AND -DEPENDENT INTERACTIONS REQUIRED FOR TISSUE FACTOR RECEPTOR AND COFACTOR FUNCTION.' cited in the application see page 2158, right column, line 29 - line 46 --- -/--	1-24

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
02 SEPTEMBER 1993	23. 09. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	RYCKEBOSCH A.O.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	FASEB JOURNAL vol. 6, no. 1, 1 January 1992, BETHESDA, MD US page A330 R.C. HAPAK ET AL. 'THE LOCATION OF THE ACTIVE SITE OF FACTOR VIIa ABOVE THE MEMBRANE SURFACE IS ALTERED BY EITHER FULL-LENGHT OR TRUNCATED TISSUE FACTOR.' see abstract nr. 1900	1-24
A	EP,A,0 225 160 (NOVO INDUSTRI A/S) 10 June 1987 see page 2, line 30 - line 38; claims	1-24
A	CHEMICAL ABSTRACTS, vol. 109, no. 17, 24 October 1988, Columbus, Ohio, US; abstract no. 142300r, A.R. GILES ET AL. 'A COMBINATION OF FACTOR Xa AND PHOSPHATIDYLCHOLINE-PHOSPHATIDYLSERINE VESICLES BYPASSES FACTOR VIII IN VIVO.' page 42 ; see abstract & BR. J. HAEMATOL. vol. 69, no. 4, 1988, pages 491 - 497 cited in the application	1-24

INTERNATIONAL SEARCH REPORT

International application No
PCT/US93/04493

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.
because they relate to subject matter not required to be searched by this Authority, namely

Remark: Although claim 1-36 are directed to a method of treatment of the human/animal body the research has been carried out and based on the alleged effects of the compound/composotion.

2. [ ] Claims Nos.
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9304493
SA 74681

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 02/09/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0225160	10-06-87	AU-B- 593042	01-02-90
		AU-A- 6567086	28-05-87
		DE-A- 3680994	26-09-91
		US-A- 5180583	19-01-93
		JP-A- 62195335	28-08-87

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82