METHOD FOR TREATMENT OF INDWELLING CATHETER OCCLUSION USING FIBRINOLYTIC METALLOPROTEINASES

This application is a continuation-in-part of U.S. Application No. 09/466,276, filed December 17, 1999, which is hereby incorporated by reference.

Field of the Invention

This invention relates to the therapeutic administration of fibrinolytic metalloproteinases, and more specifically to a method for administering such agents *in vivo* via localized delivery to vascular thrombi in order to effect clot lysis. This invention also relates to a method for the dissolution of blood clots in an indwelling catheter, shunt or other vascular access device in order to restore function to the vascular access device.

Background of the Invention

Vascular occlusions caused by blood clots such as thrombi and embolisms are serious medical maladies that can become limb or life threatening if not timely treated. Devices and methods have been developed for the treatment and removal of vascular blood clots. By way of illustration, see U.S. Patent No. 4,447,236 (Quinn), issued May 8, 1984; U.S. Patent No. 4,692,139 (Stiles), issued September 8, 1987; U.S. Patent No. 4,755,167 (Thistle et al.), issued July 5, 1988; U.S. Patent No. 5,167,628 (Boyles), issued December 1, 1992; U.S. Patent No. 5,222,941 (Don Michael), issued June 29, 1993; U.S. Patent No. 5,250,034 (Appling et al.), issued October 5, 1993: U.S. Patent No.5,370,653

(Cragg), issued December 6, 1994; U.S. Patent No.5,380,273 (Dubrul et al.), issued January 10, 1995; U.S. Patent No. 5,498,236 (Dubrul et al.), issued March 12, 1996; U.S. Patent No. 5,626,564 (Zhan et al.), issued May 6, 1997; U.S. Patent No. 5,709,676 (Alt), issued January 20, 1998; U.S. Patent No. 5,865,178 (Yock), issued February 2, 1999, and WO 90/07352 (published July 12, 1990). Such methods and devices include infusion catheters for delivering thrombolytic or fibrinolytic agents to the blood clot and dissolving it. Infusion catheters are typically used in conjunction with enzymatically active agents that are capable of degrading the fibrin in the clot and thus effectively dissolving the clot. Such enzymes are typically referred to as "thrombolytic" or "fibrinolytic" agents.

Fibrolase is a known fibrinolytic zinc metalloproteinase that was first isolated from the venom of the southern copperhead snake (*Agkistrodon contortrix contortrix*). See Guan et al., Archives of Biochemistry and Biophysics, Volume 289, Number 2, pages 197-207 (1991); Randolph et al., Protein Science, Cambridge University Press (1992), pages 590-600; European Patent Application No. 0 323 722 (Valenzuela et al.), published July 12, 1989; and United States Patent No. 4,610,879 (Markland et al.), issued September 9, 1986. Fibrolase has been shown to be fibrinolytic, and this metalloproteinase has been documented to have proteolytic activity against the fibrinogen Aα-chain, with reduced proteolytic cleavage of the Bβ-chain and no activity against the γ-chain of fibrinogen; Ahmed et al., Haemostasis, Volume 20, pages

147-154 (1990). Because fibrin is a principal component of blood clots, the fibrinolytic properties of fibrolase point to its potential as a clot dissolving agent for *in vivo* thrombolytic use; see Markland et al., Circulation, Volume 9, Number 5, pages 2448-2456 (1994), and Ahmed et al., above.

Novel Acting Thrombolytic (NAT) is a modified form of fibrolase that differs from fibrolase in that NAT contains 201 amino acids with an N-terminal sequence of SFPQR, whereas the N-terminal sequence of native fibrolase begins with EQRFPQR and is 203 amino acids in length. The amino-terminal modification was designed to prevent chemical reactions at amino acid residues that were capable of forming a variable quantity of cyclized glutamine (pyroglutamic acid) which have the potential to create lot-to-lot variations in quality and uniformity of the product. Thus, NAT can be viewed as a more stable molecule.

Despite these structural differences, NAT and fibrolase are similar with respect to enzymatic (fibrinolytic) activity. This similarity in biological activity is consistent with data indicating that the active site of the fibrolase molecule spans amino acids 139-159, as described by Manning in Toxicon, Volume 33, pages 1189-1200 (1995), and its predicted location in three-dimensional space is distant from the amino-terminus. The active site of the fibrolase and NAT molecules contains a zinc atom which is complexed by three histidine residues.

Published literature on venom-derived fibrolase has demonstrated its proteolytic activity against fibrinogen at the Lys^{413}-Leu^{414} site and against the oxidized β-chain of insulin at the Ala^{14}-Leu^{15} site; Retzios and Markland, Thrombosis Research, Volume 74, pages 355-367 (1994); Pretzer et al., Pharmaceutical Research, Volume 8, pages 1103-1112 (1991), and Pretzer et al., Pharmaceutical Research, Volume 9, pages 870-877 (1992). NAT has also been determined to have proteolytic activity on these substrates at the same cleavage sites.

In contrast to fibrinolytic metalloproteinases such as fibrolase and NAT, clot lysing agents such as streptokinase, urokinase and tissue-type plasminogen activator (tPA) are plasminogen activators which promote thrombolysis by activation of the endogenous fibrinolytic system. More specifically, plasminogen activators catalyze the conversion of plasminogen into plasmin, a serine protease. Plasmin is capable of cleaving fibrinogen and fibrin at arginyl-lysyl bonds, and it is through the generation of plasmin that the plasminogen activators ultimately affect fibrin degradation and clot lysis. Current commercially available thrombolytic agents are plasminogen activators, such as urokinase, streptokinase or tPA.

Unlike the plasminogen activator class of thrombolytic drugs, fibrinolytic metalloproteinases, such as fibrolase and NAT, do not rely on the endogenous fibrinolytic system (conversion of plasminogen to plasmin). Hence, this class of clot lysing agents can be distinguished from the plasminogen

activators by their unique mode of action and are defined as "direct" fibrinolytic agents.

Alpha$_2$-macroglobulin is a prevalent proteinase inhibitor present in mammalian serum and is one of the largest of the serum proteins (having a molecular weight of 725 kilodaltons). The specificity of α_2-macroglobulin for proteinases is broad, encompassing serine, cysteine, aspartic and metalloproteinase classes. The α_2-macroglobulin molecule is a tetramer of identical subunits that are disulfide bonded in pairs with a non-covalent association of the half molecules. Thus, under reducing conditions, native α_2-macroglobulin can be dissociated into its four monomeric subunits.

Each subunit of α_2-macroglobulin possesses a region that is very susceptible to proteolytic cleavage (the "bait" region). Proteolysis of the bait region induces a conformational change in α_2-macroglobulin, which entraps the proteinase within the α_2-macroglobulin molecular structure. This process is described in the literature as a "venus fly-trap" interaction. Once the proteinase is entrapped, it is sterically hindered and therefore cannot access its macromolecular substrate.

In addition, a covalent bond can form between α_2-macroglobulin and many of the proteinases that it entraps. As mentioned, entrapment of a proteinase induces a conformational change in the α_2-macroglobulin molecule. It is presumed that upon this conformational

change, a thioester bond on the interior of the α_2-macroglobulin molecule becomes reactive and can form a covalent bond with nucleophilic residues (such as lysine) of the entrapped proteinase. Thus, within the general circulation, α_2-macroglobulin can effectively neutralize a variety of proteinases.

Moreover, the conformational change in α_2-macroglobulin brought about by the entrapment of a proteinase results in a form that is recognized by the reticuloendothelial system. Clearance of α_2-macroglobulin-entrapped proteinases is generally described with half-life values in minutes and is believed to occur through the low-density lipoprotein (LDL)-receptor related protein expressed on macrophages, hepatocytes and fibroblasts. For more on α_2-macroglobulin, see Methods in Enzymology, edited by A.J. Barrett, Academic Press, Inc., Philadelphia, (1981), pages 737-754.

Alpha$_2$-macroglobulin is capable of forming a macromolecular complex with fibrolase, NAT and other proteinases. Unlike some proteinases that can form a dissociable complex with α_2-macroglobulin, fibrolase and NAT are two examples of fibrinolytic metalloproteinases that form a complex which cannot be dissociated from α_2-macroglobulin under physiologic conditions. When purified human α_2-macroglobulin and NAT, for instance, are incubated together, formation of the complex begins in seconds and is nearly complete within a few minutes. This phenomenon shows that *in vitro* complex formation can be rapid and is suggestive

of the potential rapidity of complex formation between α_2-macroglobulin and NAT or other fibrinolytic metalloproteinases *in vivo*.

Although α_2-macroglobulin is one of the major plasma proteins, there is nonetheless a finite quantity of α_2-macroglobulin in the circulation that would be available to bind and neutralize a fibrinolytic metalloproteinase. The α_2-macroglobulin binding capacity is therefore saturable. Once the α_2-macroglobulin binding capacity has been exceeded, the concentration of unbound fibrinolytic metalloproteinase rises proportionally as additional fibrinolytic metalloproteinase is added to the sample.

The presence of α_2-macroglobulin in the general circulation of a patient presents a challenge for the systemic (for example, intravenous) administration of fibrolase, NAT and other fibrinolytic metalloproteinases that are bound up by α_2-macroglobulin in the general blood circulation. Unless the saturable level of innate α_2-macroglobulin is exceeded by the systemically administered dose of such fibrinolytic metalloproteinases, the latter will effectively be neutralized and rendered ineffective for therapeutic purposes.

In one *in vivo* study, conducted in rabbits, the biological effectiveness of venom-derived fibrolase was examined following systemic intravenous administration. Ahmed et al., Haemostasis, above. The dose of fibrolase used was 3.7 milligrams per kilogram, which

was estimated to yield a final blood concentration of approximately 60 micrograms per milliliter in a 3.0-kilogram rabbit. This amount was chosen based on studies examining the inactivation of the enzyme in the presence of blood or plasma, presumably due to α_2-macroglobulin (see pages 336 and 339).

In another *in vivo* study, the biological effect of recombinant fibrolase on clot lysis was examined in canines. Markland et al., Circulation, above. Four milligrams of this material per kilogram (of animal body weight) was infused over five minutes proximal to a pre-induced thrombus in the left carotid artery via a catheter device (see page 2450). Here again, it was noted that inactivation of fibrolase occurs in the general blood circulation presumably due to the presence of α_2-macroglobulin (see page 2454, second column, last paragraph).

As these two studies show, the deactivating effects of α_2-macroglobulin can be overcome by either administration or systemic dosages of fibrinolytic metalloproteinase that exceed the saturable level of innate α_2-macroglobulin (the rabbit study) or by delivering the enzyme locally to the site of the clot (the dog study) and avoiding systemic administration. On the other hand, doses of the fibrinolytic metalloproteinase in excess of the saturable level of α_2-macroglobulin, whether delivered systemically or locally, may exceed levels that are safe and well tolerated by the subject being treated. Notably, fibrinolytic metalloproteinases are capable of

destroying not only fibrin, but they may also degrade other structural proteins and are therefore potentially toxic *in vivo* when present in large amounts that exceed the saturable level of α_2-macroglobulin.

The formation of a blood clot or other fibrin-based occlusion is also a concern when using an indwelling catheter or other vascular access device. There are many conditions that require recurrent or prolonged use of a vascular access device, such as a catheter, access graft, sheath, needle, arteriovenous fistula or shunt. For example, various procedures associated with hemodialysis, chemotherapy, blood infusion or exchange and other procedures involving recurrent intravenous or intraartieral drug delivery (or fluid withdrawal) may involve the use of indwelling catheter or other permanent or semi-permanent implanted medical device. Blood clots may form in or around, or attach to, any device that has been introduced into a vascular space, particularly where the device remains in the vascular space for an extended period of time or where the device has one or more very small openings. In addition, other fibrin-based occlusions may attach to any portion of an indwelling vascular access device which could prevent or hinder the proper functioning of the device.

It would be useful to have a fast and efficient method to treat, i.e., destroy, dissolve or lyse clots or other occlusions that have formed in, around or attached to, an indwelling vascular access device. In the absence of an efficient method to treat clots that have formed in or around a vascular access device, the

indwelling device may have to be replaced by a physician, incurring additional cost and risk to the patient.

Accordingly, it is an object of the present invention to provide a safe and effective method for treatment of a blood clot or occlusion in or around an indwelling vascular access device.

It is also an object of the present invention to provide a method for restoring patency to a fully or partially occluded indwelling vascular access device.

It is a further object of the present invention to provide a method for restoring function to an indwelling vascular access device where function has been altered by a fibrin-based occlusion.

It is also an object of the present invention to provide a safe and biologically effective way of using locally administered fibrinolytic metalloproteinases to lyse blood clots *in vivo*.

Summary of the Invention

In a first aspect of the present invention, there is provided a use of a fibrinolytic matalloproteinase selected from the group consisting of fibrolase and Novel Acting Thrombolytic (NAT) in the manufacture of a composition for lysing a blood clot in, on, or attached to an indwelling vascular access device to restore patency to the indwelling vascular access device.

In a second aspect of the present invention, there is provided a use of a fibrinolytic metalloproteinase selected from the group consisting of fibrolase and Novel Acting Thrombolytic (NAT) in the manufacture of a composition for lysing a blood clot in, on, or attached to an indwelling vascular access device to restore function to the indwelling vascular access device.

In certain embodiments, this invention is a method for the therapeutic treatment of a blood clot in an indwelling vascular access device by administration through said vascular access device of a quantity of fibrinolytic metalloproteinase in a pharmaceutically acceptable solution, wherein the quantity does not exceed 1.7 milligrams of fibrinolytic metalloproteinase per kilogram of body weight of the human subject being

treated. In certain embodiments, the vascular access device is indwelling in an artery or vein of a human subject. In certain embodiments, the indwelling vascular access device is a catheter, shunt, access graft, needle or sheath. In certain embodiments, the vascular access device is employed to introduce or remove fluids from a vascular area. In certain embodiments, the vascular access device of this invention is used in connection with hemodialysis, blood transfusion, removal or exchange, chemotherapy or any other procedure that requires the introduction or removal of fluids from a vein or artery.

In certain embodiments, this invention is a method for lysing blood clots in an indwelling vascular access device by local administration to said clot of a quantity of fibrinolytic metalloproteinase that complexes with alpha-two macroglobulin in an amount sufficient to facilitate lysing of the clot while not exceeding a level significantly above saturable level of alpha-two macroglobulin in said human subject, in a pharmaceutically acceptable solution.

In certain embodiments, this invention is a method for restoring patency to a fully or partially occluded indwelling vascular access device. In certain embodiments, this invention is a method for restoring function to an indwelling vascular access device.

Brief Description of the Figures

FIGURES 1A-1C. Figure 1A is a baseline angiogram of the carotid artery in an adult pig prior to balloon

catheter induced-injury and the formation of an occlusive thrombus Figure 1B is an angiogram taken on day 4 in the same animal, prior to the administration of NAT in accordance with the method of this invention. Figure 1C is an angiogram at 2 hours following administration of 30 mg of NAT through a "PRO" catheter (see Figure 3 for an illustration of this device).

FIGURE 2 illustrates a type of catheter device designed for localized delivery of a thrombolytic agent in a blood vessel.

FIGURE 3 is a side cross-sectional view of an alternate type of catheter device for localized delivery of a thrombolytic agent in a blood vessel.

FIGURE 4 is a histogram of estimated maximum dose of NAT in patients with peripheral vascular occlusion.

FIGURE 5 illustrates the use of NAT in an *in vitro* catheter occlusion model.

FIGURE 6 illustrates summary results of numerous experiments, the resulting mean data showing that NAT resolves in vitro occlusions up to 40% faster than Abbokinase® Open Cath®, a commercial urokinase product for catheter clearance.

Detailed Description of the Invention

In certain embodiments this invention is a method for the treatment of a blood clot *in vivo*, in human subjects, by a fibrinolytic metalloproteinase,

comprising locally administering a safe, biologically effective amount of the fibrinolytic metalloproteinase to the blood clot, such as by use of a catheter or other vascular access device. Typically, stationary fibrin clots will be located in a blood vessel (arterial or venous, native or synthetic (e.g., a graft)) in a human subject. In certain embodiments, this invention is a method for treating a blood clot located in, around or attached to an indwelling catheter, shunt, needle or other vascular access device. In certain embodiments, this invention is a method for restoring patency to a fully or partially occluded indwelling vascular access device. In certain embodiments, this invention is a method for restoring function to a vascular access device where function has been altered or compromised to some extent by a fibrin-based occlusion.

By "safe, biologically effective" amount is meant an amount sufficient to degrade fibrin and facilitate lysing of the clot (i.e., thrombus), while not exceeding levels significantly above the saturable level of α_2-macroglobulin in the circulatory system of the patient being treated (i.e., levels that may cause damage to blood vessel walls). Typically, this amount will be in the range between 0.025 and 1.7 milligrams per kilogram of body weight for the human subject being treated, as determined from a study conducted with blood samples from human subjects that have been studied for *in vitro* α_2-macroglobulin content and binding capacity. From the *in vitro* results of this study, it has been possible to define the saturable level *in vivo* of α_2-macroglobulin for all practical

purposes, thus enabling the delineation of a biologically effective amount that takes into account not only the minimum level of fibrinolytic metalloproteinase required for biological effectiveness, but also the maximum level for well-tolerated administration. This study is described in detail further below among the Examples.

The terms "locally" or "localized" as applied to the manner of delivery of the fibrinolytic metalloproteinase herein refers to intra-arterial or intravenous administration either directly to the blood clot itself (i.e., intrathrombus) or in close proximity to the clot (either proximal or distal relative to blood flow) and near enough for the majority of the fibrinolytic metalloproteinase to be absorbed by the clot. For the treatment of clots in, around or attached to a vascular access device, or to restore patency or function to vascular access device, delivery of a fibrinolytic metalloproteinase is generally effected via the vascular access device itself, and is therefore inherently local. In certain embodiments, a secondary vascular access device, such as a catheter, may be used to deliver a fibrinolytic metalloproteinase to an implanted medical device, such as a stent.

The term "catheter delivery means" or "vascular access device" is employed herein in the conventional sense of referring to a tubular medical device for insertion into canals, vessels, passageways or body cavities for the purpose of injecting or withdrawing fluids or to keep a passageway open. In general, such means will typically comprise an elongated flexible

catheter body containing one or more interior passageways (or "lumens"); a proximal portion which allows material (i.e., clot lysing agent) to be introduced into the catheter body and to flow through the lumen; a distal portion optionally having a tapered end; and one or more exit ports at or near the end of the distal portion to permit material to exit the catheter in response to applied pressure.

The terms "proteolytic degradation," dissolution," "lysis" and "therapeutic treatment" are all used herein to refer to the degradation, disintegration, decomposition, break up or other removal of a blood clot or other fibrin-based occlusion. The blood clot may partially or totally occlude the lumen of a vessel or medical device. Similarly, "restoring patency " refers to any measurable increase in blood flow (for example, by volume or speed) through a vessel or device lumen or exit port due to the dissolution of a blood clot therein.

The term "blood clot" or "occlusion" as used herein refers to any fibrin-based mass, cluster, obstruction or growth. Typically blood clots result when blood coagulates in an artery or vein and impede, obstruct, hinder or block, totally or partially, the flow of blood therethrough. Blood clots can also be formed in or around any foreign object introduced into a vascular space. Blood clots can be stationary, such as a thrombus (a blood clot that forms in a vessel and remains there) or it may be transportable, such as an embolism (blood clots that travels from the site where it formed to another location in the body). Blood

clots can also vary widely in size, shape and composition, and can include spherical masses as well as flaps or other planar structures. Sometimes, a piece of atherosclerotic plaque, small pieces of tumor, fat globules, air, amniotic fluid or other materials found in the blood can act in the same manner as a blood clot. To the extent such masses contain fibrin or other substances susceptible to degradation by a fibrinolytic metalloproteinase, one method of the present invention would be useful for the treatment thereof.

Another method of this invention is illustrated further below with respect to peripheral arterial occlusion (PAO). PAO finds its origins in peripheral vascular disease due to atherosclerosis. The symptoms develop slowly over many years as atherosclerosis progresses, with a critical ischemic level being reached in about 15 to 20% of patients with lower extremity disease. Medical therapy is limited and predominantly aimed at prevention or risk reduction using medications such as lipid-lowering or antiplatelet agents, smoke cessation programs and physical exercise. Jackson and Clagett, Chest, Volume 114, pages 666S-682S (1998).

The clinical manifestations of peripheral vascular disease may include acute occurrences of limb-threatening ischemia or the presence of more chronic evidence of vascular disease (i.e., intermittent claudications). Outside of the aforementioned preventive measures, chronic PAO is typically not treated until the onset of severe lifestyle limitation

or limb-threatening ischemia. Depending on the vessel segment affected and the extent of occlusion, available medical interventions include percutaneous transluminal angioplasty, surgical revascularization, and thrombolysis. Studies have shown that the intra-arterial infusion of clot lysing agents, particularly in the early stages of occlusion, can avoid the need for surgical intervention. As demonstrated in the Rochester trial, which compared thrombolysis with the plaminogen activator urokinase to surgery in the treatment of acute PAO (Ouriel et al., Journal of Vascular Surgery, 1994, Volume 19, pages 1021-1030), approximately 33 percent of patients in the thrombolysis arm of the study were successfully treated with medical intervention only, thereby avoiding a more invasive procedure. In contrast, 98 percent of patients in the operating arm were subjected to an endovascular or surgical procedure.

Other medical disorders involving occlusive blood clots can be effectively treated in a similar manner by the present method, including, but not limited to, acute myocardial infarction, ischemic stroke, deep venous thrombosis and pulmonary embolism. In certain embodiments, the method of this invention can also be employed to dissolve clots which occur with chronically implanted medical devices such as indwelling catheters and hemodialysis access grafts. Other exemplary implanted medical devices include shunts, access parts, needles, sheaths or other vascular access devices.

In certain embodiments, this invention is applicable for the therapeutic delivery of any

fibrinolytic metalloproteinase that is capable of being complexed with α_2-macroglobulin. Such fibrinolytic metalloproteinases, if naturally occurring, may be purified from their natural sources, e.g., fibrolase from snake venom. Alternatively, polypeptide fibrinolytic metalloproteinase agents the nucleic acid and amino acid sequences of which are known may be produced by utilizing conventional methods of recombinant expression and purification.

In general, recombinant methods employ a DNA molecule encoding the fibrinolytic metalloproteinase of interest which is inserted into an appropriate vector for expression in a suitable host cell. The vector is selected to be functional in the particular host cell employed, i.e., is compatible with the host cell machinery, such that expression of the DNA can occur. The vectors may also contain a 5' flanking sequence (also referred to as a "promoter") and other expression regulatory elements operatively linked to the DNA to be expressed, as well as other known elements, such as an origin of replication element, a transcriptional termination element, a complete intron sequence containing a donor and acceptor splice site, a signal peptide sequence, a ribosome binding site element, a polyadenylation sequence, a polylinker region for inserting the encoding nucleic acid, and a selectable marker element. The vector may also optionally contain a "tag" sequence, i.e., an oligonucleotide sequence located at the 5' or 3' end of the polypeptide-coding sequence that encodes polyHis or another small immunogenic sequence. This tag will be expressed along with protein of interest, and can serve as an affinity tag for purification of this polypeptide from the host cell. If desired, the tag can subsequently be removed

from the purified polypeptide by various means, for example, with use of a selective peptidase.

In those cases where it is desirable for the polypeptide to be secreted from the host cell, a signal sequence may be used to direct the polypeptide out of the host cell where it is synthesized. Typically, the signal sequence is positioned in the coding region of nucleic acid sequence, or directly at the 5' end of the coding region. Many signal sequences have been identified, and any which are functional in the selected host cell may be used.

After the vector has been constructed and a nucleic acid has been inserted into the proper site of the vector, the completed vector may be inserted into a suitable host cell for amplification and/or polypeptide expression. Host cells may be prokaryotic (such as *E. coli*) or eukaryotic (such as a yeast cell, an insect cell, or a vertebrate cell).

Suitable host cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO) or 3T3 cells. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. Other suitable mammalian cell lines are the monkey COS-1 and COS-7 cell lines, and the CV-1 cell line. Further exemplary mammalian host cells include primate cell lines and rodent cell lines, including transformed cell lines. Normal diploid cells, cell strains derived from *in vitro* culture of primary tissue, as well as primary explants, are also suitable. Candidate cells may be genotypically deficient in the selection gene, or may contain a dominantly acting selection gene. Still other suitable

mammalian cell lines include but are not limited to, HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines.

Also useful as host cells are bacterial cells, for example, various strains of *E. coli, and various* strains of yeast cells.

Insertion (also referred to as "transformation" or "transfection") of the vector into the selected host cell may be accomplished using such methods as calcium phosphate, electroporation, microinjection, lipofection or the DEAE-dextran method. The method selected will in part be a function of the type of host cell to be used. These methods and other suitable methods are well known to the skilled artisan.

The host cell, when cultured under appropriate conditions, can synthesize the fibrinolytic metalloproteinase of interest. The host cells may be cultured using standard media well known to the skilled artisan. The media will usually contain all nutrients necessary for the growth and survival of the cells. Suitable media for culturing *E. coli* cells are, for example, Luria Broth (LB) and/or Terrific Broth (TB). Suitable media for culturing eukaryotic cells are RPMI 1640, MEM, DMEM, all of which may be supplemented with serum and/or growth factors as required by the particular cell line being cultured.

Typically, an antibiotic or other compound useful for selective growth of the transformed cells only is added as a supplement to the media. The compound to be used will be dictated by the selectable marker element present on the plasmid with which the host cell was transformed. For example, where the selectable marker

element is kanamycin resistance, the compound added to the culture medium will be kanamycin.

The amount of protein produced in the host cell can be evaluated using standard methods known in the art, including Western blot analysis, SDS-polyacrylamide gel electrophoresis, non-denaturing gel electrophoresis, HPLC separation, immunoprecipitation, and/or activity assays such as DNA binding gel shift assays.

If the protein is secreted from the host cells other than gram-negative bacteria, the majority will likely be found in the cell culture medium. If it is not secreted, it will be present in the cytoplasm. For intracellular protein, the host cells are typically first disrupted mechanically. For protein having a periplasmic location, either mechanical disruption or osmotic treatment can be used to release the periplasmic contents into a buffered solution, and the polypeptide is then isolated from this solution. Purification from solution can thereafter be accomplished using a variety of techniques.

If the protein has been synthesized so that it contains a tag such as hexahistidine or other small peptide at either its carboxyl or amino terminus, it may essentially be purified in a one-step process by passing the solution through an affinity column where the column matrix has a high affinity for the tag or for the polypeptide directly (i.e., a monoclonal antibody). Where the polypeptide has no tag and no antibodies are available, other well known procedures for purification can be used, for example, ion exchange chromatography, molecular sieve chromatography, reversed phase chromatography, HPLC, native gel

electrophoresis in combination with gel elution, and preparative isoelectric focusing ("Isoprime" machine/technique, Hoefer Scientific). In some cases, two or more of these techniques may be combined to achieve increased purity.

Novel Acting Thrombolytic (NAT) polypeptide utilized herein to illustrate the practice of this invention refers in general to the fibrinolytically active metalloproteinase of SEQ ID NO: 1. The NAT polypeptide is encoded by the cDNA molecule of SEQ ID NO: 2, although any DNA molecule of variant sequence encoding the same polypeptide may be used for expression and manufacture in accordance with specific methods which are referred to further below.

Fibrolase has been described in the scientific and patent literature; see references above. Typically, the form of fibrolase which is employed in the practice of this invention will be of SEQ ID NO: 3, which is encoded by the cDNA molecule of SEQ ID NO: 4 or variants thereof encoding the same amino acid sequence.

Preferably, a yeast expression system is employed for recombinant expression of NAT. Special mention is made of Pichia strains, for example, *Pichia pastoris*, as being the most advantageous and preferred for use. A detailed description of such a system may be found in United States Patent No. 4,855,231 (Stroman et al.), United States Patent No. 4,812,405 (Lair et al.), United States Patent No. 4,818,700 (Cregg et al.), United States Patent No. 4,885,242 (Cregg), and United States Patent No. 4,837,148 (Cregg), the disclosures of which are hereby incorporated by reference. Expression of fibrolase in such a system will typically involve a DNA molecule of SEQ ID NO: 5, which encodes "prepro"

sequence (nucleotides 1-783) in addition to the "mature" polypeptide (nucleotides 784-1392). The expression of NAT in such a system will typically involve a DNA molecule of SEQ ID NO: 6, which encodes "prepro" sequence (nucleotides 1-783) in addition to the "mature" polypeptide (nucleotides 784-1386).

The fibrinolytic metalloproteinase employed in accordance with this invention, whether it be NAT, fibrolase, or some other fibrinolytic metalloproteinase, is administered in the form of a pharmaceutically acceptable solution, alone or containing additional pharmaceutically acceptable ingredients. If desired, such solutions may comprise, in addition to the fibrinolytic metalloproteinase and a solvent (i.e., distilled water or physiological saline), standard ingredients such as stabilizers (to prevent protein aggregation or physical or chemical degradation in aqueous media), bulking agents (to provide bulk), diluents, antibacterial agents, viscosity adjusting agents, anti-oxidants, and so forth, in conventional amounts. Known excipients which can be included in the formulation include polyols (including mannitol, sorbitol and glycerol); sugars (including glucose and sucrose); and amino acids (including alanine, glycine and glutamic acid). See, for example, Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania. See also WO 01/24817 A2 (the entirety of which is herein incorporated by reference) which discloses various compositions of fibrinolytic agents useful in the present invention.

Desirably, the pharmaceutical composition will be buffered (with a biocompatible buffering agent, for example, citric acid or citric acid salt) to a pH which

is at or near neutral (7.0) prior to administration, and usually between about 6.5 and about 8.0 pH (± 0.5).

If the metal ion of the fibrinolytic metalloproteinase is zinc, such as with fibrolase or NAT, it may be preferable to include a water-soluble zinc salt (for example, zinc sulfate or zinc acetate) as a stabilizer. To further enhance the long-term stability and shelf life of the composition, it may also be advantageous to freeze the solution or to convert it to a lyophilized (freeze-dried) product which is thawed or reconstituted prior to use, as the case may be.

By way of illustration, a freezable liquid medicinal composition which may be employed in the method of this invention comprises fibrolase or NAT, a water soluble zinc salt, a citric acid buffer, optionally an additional stabilizer selected from the group consisting of water soluble calcium salts, and optionally a bulking agent (for example, mannitol). A surfactant, such as Tween® 80 (BASF, Gurnee, Illinois), may also be added to increase freeze-thaw stability. Tris buffer (Sigma, St. Louis, Missouri) or another buffer with a buffer capacity above pH 7.0 may be added to stabilize the pH at or above pH 7.4. Most of these ingredients will be present in minor amounts ranging from 0.001 to 2.0 millimolar (mM) or less than ten percent (w/v). The buffering agent will be added in an amount sufficient to achieve the desired pH, and this amount may vary depending on the specific formulation.

By way of further illustration, a lyophilizable or lyophilized pharmaceutical composition which can be used in the method of this invention comprises fibrolase or NAT, a zinc stabilizer (e.g., water

soluble zinc salt such as the above), and a citric acid buffer, with or without other excipients (e.g., bulking agent such as mannitol, glycine, or the like). The lyophilized composition may also contain a disaccharide sugar, such as sucrose or trehalose, as a lyoprotectant. A surfactant, such as Tween 80®, may be added to protect against lyophilization stresses on the fibrinolytic metalloproteinase (e.g., fibrolase or NAT). The pH will ideally be maintained at pH 8.0 ± 0.5, using a suitable buffer with a pK_a in this range (for example, Tris). Amounts of ingredients will be in accordance with the above.

As mentioned, in certain embodiments, the method of this invention is employed to locally administer biologically effective amounts of a fibrinolytic metalloproteinase that are in the dose range between 0.025 and 1.7 mg/kg. Preferably, this amount will in the range from about 0.1 to about 0.5 mg/kg. Solution strengths will be formulated accordingly, with dilution to be affected as needed upon administration.

In contrast to treatment of thrombosis in a native artery or vein, such as PAO, treatment of a blood clot occurring in an implanted or indwelling vascular access device presents addition challenges. For example, in the case of an occluded central venous line, the "dead volume" of the catheter (i.e., the inner diameter of catheter multiplied by its length) defines the maximum volume that can be contained within the catheter. For example, a catheter with a 1.0 mm internal diameter and a 40.0 cm length possesses a dead volume of ~0.3 ml. Because volume can be so limited, it is important to define the proper solution strength. Accordingly,

increasing solution strength or concentration is an effective means for increasing the amount of drug available to dissolve a target blood clot.

In certain embodiments, the method of the present invention concerns the use of a fibrinolytic metalloproteinase for the treatment of occlusions associated with a vascular access device. Such clots can occur in, around or be attached to, a vascular access devices. Typically, clots will form in the lumen or exit port of a device such as a catheter, and may interfere with the function of the catheter. Clots can also occur on the exterior of a vascular access device, such as a flap that may cover an exit port and thereby prevent the introduction of a drug or removal of blood. Clots can also attach to a vascular access device, interfering with the proper functioning of the device. The interference can result from a partial occlusion or blockage, i.e., some fluid may pass through, or a total occlusion in which no fluid can pass.

To demonstrate one method of the present invention, an in vitro catheter occlusion model was used to simulate a clot in an indwelling catheter-type device. In this model, collagen-coated Pasteur pipettes were used to simulate an indwelling portion of a catheter. Human blood was drawn via a finger stick by capillary action to a length of 3.0 mm and allowed to clot. The tip of one pipette was introduced into a vial containing saline and another into a 4mg of NAT (100ul of a 40mg/ml solution strength) solution of NAT. Figure 5 illustrates the results of this in vitro

catheter occlusion model, which indicate that the clot in the saline is still intact, while the clot in 40mg/ml is active in dissolving the clot.

In another experiment, a separate group of pipettes (each containing a blood clot at its tip) was introduced into and incubated in a vial containing the following: 100ul of saline, 100ul of a urokinase solution (5000 IU/ml) and 100ul of a NAT solution where the quantity of drug was varied from 0.5 to 4mg by increasing the solution strength from 5mg/ml to 40mg/ml. Figure 6 illustrates summary results of numerous experiments, the resulting mean data showing that NAT resolves in vitro occlusions up to 40% faster than Abbokinase® Open Cath®, a commercial urokinase product for catheter clearance (second column, UK 500 I.U.) Figure 6 also demonstrates the dose-dependent nature of NAT in terms of the speed at which NAT acts to proteolytically degrade a blood clot.

For treatment of a relatively small indwelling catheter occlusion, such as that simulated by the in vitro catheter occlusion model, a preferred range for treatment of would be approximately 5 to 40mg/ml of fibrolase, NAT or other fibrinolytic metalloproteinase. However, depending on the type and size of the vascular access or other medical device, and the position, size and extent of the clot or occlusion, much smaller or larger doses may be required. For example, a clot in a relatively large hemodialysis shunt would likely require a higher concentration of fibrinolytic metalloproteinase than a clot blocking the exit port of a 1.0mm diameter catheter. Accordingly, concentrations

for this indication may range anywhere from 0.1 mg/ml or less, to over 80 mg/ml.

For blood clots located in, around or attached to an indwelling vascular access device, an effective dose can be delivered in any number of ways, including pulsatile infusion, continuous infusion, bolus administration, or a combination of all three. Given the difficulty posed by "dead volume" constraints, a bolus administration is generally preferred.

In the typical case, the method of this invention is carried out in conjunction with a catheter-directed thrombolysis procedure. Such procedures involve the use of a pre-sterilized catheter-type drug delivery device, the side walls of which may be made of a thin, semi-rigid or flexible biocompatible material (for example, a polyolefin, fluoropolymer, or other inert polymer). In general, suitable catheters contain at least one interior cavity (or lumen) running the length of the device. The material from which the catheter is constructed is flexible enough to be moved through the interior of the vasculature without causing injury to the blood vessel walls, yet sufficiently rigid to extend over a distance to the site of treatment while the interior cavity of the device remains fully distended. Typically, such catheter devices will range from 2 to 20 on the French scale for catheter diameters (1/3 millimeter equals 1 French) and from two to six feet or more in length.

Exemplary catheter devices for the intravascular delivery of thrombolytic medication in accordance with this invention are illustrated in Figures 2 and 3, the practical applications of which are described in detail

in the examples below. However, any conventional catheter delivery or vascular access device which is suitable for this method may be utilized, including but not limited to the specific devices referred to herein.

For example, Figure 2 illustrates a type of catheter device designed for localized delivery of a thrombolytic agent in a blood vessel. The device, shown in side cross-sectional view, contains "side holes" at the delivery end through which the infusate (thrombolytic agent) is emitted under applied fluid pressure. The diameters of the catheter tubes in this figure and the following figure relative to overall size are exaggerated to show the details better. See also Figure 3, which illustrates a side cross-sectional view of an alternate type of catheter device for localized delivery of a thrombolytic agent in a blood vessel. This device contains thin slits, referred to as "pressure response outlets" (PRO), which have been cut into the catheter wall at regular intervals such that infusate escapes when the fluid pressure within the catheter reaches a critical point, causing the slit to open. The device can be used in conjunction with an automated, piston-driven, pulsed infusion device (not shown, but exemplified further below) which is capable of delivering pulses of drug infusion.

For blood clots that occur in a vein or artery and are not associated with the introduction of a vascular access or other medical device, an effective dose of fibrinolytic metalloproteinase can be delivered through the catheter to the local site of treatment by pulsatile infusion, continuous infusion, bolus administration, or a combination of all three. The solution strength (i.e., concentration) of fibrinolytic

metalloproteinase in the treatment solution is also an important parameter for these types of clots. More specifically, a range between the minimum dilution of the fibrinolytic metalloproteinase for effectiveness at the lower end (which is especially important for bolus administration) and the maximum solubility of the fibrinolytic metalloproteinase at the higher end should be selected. In general, solution strengths in the range from about 0.1 to about 80 mg/ml are employed. The volume of the bolus (or total volume of multiple boluses in the case of a "pulsed" delivery) is then selected accordingly to deliver an effective amount of fibrinolytic metalloproteinase within the ranges prescribed above.

Description of Specific Embodiments

The invention is further illustrated in the following examples, which are meant to be illustrative only and not to limit the invention to the described embodiments. In these examples, and throughout the description of this invention, "kg" indicates kilograms of body weight per test subject, "mg" indicates milligrams, "mL" indicates milliliters, and "min" indicates minutes. The fibrinolytic metalloproteinase illustrated, namely Novel Acting Thrombolytic (NAT), was recombinantly-derived and made in accordance with methods referred to above.

EXAMPLE 1

Thrombolysis in Subacute Thrombosis of the Adult Pig Common Carotid Artery

NAT was been studied in a model of subacute thrombosis of the carotid artery in adult pigs, averaging 75 kg in body weight, at a contract laboratory (Charles River Laboratories, Southbridge, Massachusetts). The intent of this study was to establish the fibrinolytic activity of NAT in a thrombosis model which is relevant to peripheral artery occlusion in humans.

In this animal model, the carotid artery was thrombosed along its entire length (approximately 20 centimeters from origin at the aorta to the carotid bifurcation) by a combination of balloon injury, thrombin and stasis. The size of the thrombus approaches the size of thrombus encountered clinically in humans with peripheral arterial occlusion. After successful thrombosis, the animal was allowed to recover for a period of four days. A four-day period was selected to allow extensive cross-linking of fibrin, remodeling of thrombus, and infiltration of cells. Notably, both the size and age of the thrombus in this model are reasonable representations of the size and duration of ischemic symptoms reported in the most recently published TOPAS trial of thrombolysis with plasminogen activators in peripheral arterial occlusion in humans. For reference, see Ouriel et al., New England Journal of Medicine, Volume 338, pages 1105-1111 (1998).

Briefly, the common carotid artery can be thrombosed along its entire length by fluoroscopically directed balloon injury. The balloons that are used are oversized and non-compliant. The balloons are inflated at pressures of up to twelve atmospheres,

which causes a crushing injury to the intimal layer of the vessel. While the balloon is inflated, it is moved back and forth in order to strip away the vascular endothelium.

The ballooning procedure is very injurious and creates a highly thrombogenic vessel surface and is repeated throughout the entire length of the common carotid artery. After thoroughly injuring the entire artery, the balloon is withdrawn to a proximal position near the aorta and inflated to occlude flow of blood through the vessel. While occluded, fifty Units of bovine thrombin is injected through the distal port of the balloon catheter in order to stimulate coagulation. The balloon remains inflated for a period of thirty minutes, which results in thrombotic occlusion of the vessel. After thirty minutes, the balloon is deflated and an angiogram is performed to confirm that the vessel has become occluded. With these procedures, occlusion of the vessel was achieved in greater than ninety percent of cases.

Figure 1A is a baseline angiogram of the carotid artery in an adult pig prior to balloon catheter induced-injury and the formation of an occlusive thrombus. The arrow indicates the position and the presence of contrast media in the left common carotid artery, indicating that blood flow in the artery is unobstructed (i.e., the blood vessel is "patent" or has ""). Figure 1B is an angiogram taken on day 4 in the same animal, prior to the administration of NAT in accordance with the method of this invention. The arrow indicates the position of the left common carotid artery, however, contrast media does not flow in the

artery due to the presence of an occlusive thrombus. Figure 1C is an angiogram at 2 hours following administration of 30 mg of NAT through a "PRO" catheter (see Figure 3 for an illustration of this device). The arrow indicates the presence of contrast media in the vessel, demonstrating that patency has been restored in the left carotid artery. Minimal residual thrombus is visible in the lumen of the artery.

Once thrombosed, the balloon catheter, guide catheter and access sheath are removed and the animal is allowed to recover over a period of four days. On the fourth day, the animals are re-anesthetized, the occlusion is reconfirmed, and a multiple side hole drug delivery catheter (see Figures 2 and 3) is advanced under fluoroscopic guidance and positioned such that the side holes are located within the thrombus. Thrombolysis with NAT was angiographically observed using fixed NAT dosages which ranged from 10 to 30 mg (or approximately 0.1 to 0.4 mg/kg on a weight adjusted basis), as shown in Figures 1A-1C.

As the image in Figure 1B illustrates, NAT is effective in restoring antegrade flow as assessed by angiography. To be more quantitative, flow in the target vessel is qualitatively scored according to a 4-point scale (ranging from 0 to 3) where:

0 = no flow
1 = flow estimated to be less than 30% of the contralateral (non-thrombosed) carotid
2 = flow estimated to be 30-80% of the contralateral carotid

3 = flow which is indistinguishable from the contralateral carotid

The image shown in Figure 1B was scored as flow grade equal to 3, a frequent result in thrombosed vessels treated with a fixed 30 mg dosage of NAT (approximately 0.4 mg/kg in a 75 kg pig). Tables 1-3 in the following examples below illustrate the treatment regimen and mean flow scores derived from serial angiograms. In all of the studies, respiration, body temperature, heart rate and arterial blood pressures were continually monitored and remained within physiologic ranges with no changes observed upon administration of NAT.

EXAMPLE 2

Selection of PRO Catheters and Pulse-Spray Delivery

In the clinical management of peripheral arterial occlusion, thrombolytic agents are delivered through catheters which are positioned near or embedded into the thrombus. As shown in Figures 2 and 3, there are two types of commonly used catheters.

One variety, the "side hole" catheter (Fig. 2), has tiny round side holes (2) cut into the catheter (4) near closed distal end 6, and an entry port 8 (for the solution of fibrinolytic metalloproteinase) in mating ring 10 affixed at proximal end 12. Catheter 4 is constructed of a flexible, elongated, biocompatible polymer tubing material which is hollow and thin-walled and has a uniform diameter of 2 to 20 French, and preferably 3 to 5 French. The catheter contains two

radiopaque markers 14 on the exterior surface near distal end 6 which demarcate the portion of the catheter containing side holes 2. In practice, the catheter is inserted into a surgical opening in the occluded artery or vein and, while being observed via fluoroscopy in accordance with standard approved procedures, is moved carefully through the blood vessel such that distal end 6 is positioned into or near the thrombus. Markers 14, which show up clearly on a fluoroscope image, can serve as a guide for positioning that portion of the catheter such that infusate emerging from side holes 2 will contact the thrombus directly. A pharmaceutical solution of the fibrinolytic metalloproteinase is then injected under gentle pressure from syringe-like reservoir 16 into entry port 8 and is impelled toward distal end 6, emerging through holes 2 into the thrombus, causing degradation of the fibrinous material.

When infusions of a fibrinolytic metalloproteinase are performed using this type of catheter, most of the fibrinolytic agent-containing solution tends to escape through the proximal side holes (i.e., those nearest to drug entry port 8), which has a negative impact in the uniformity of drug delivery at the site of treatment. There is also a possibility of backflow of blood into the catheter through side ports 2 under negative pressure.

Another variety of catheter, also composed of a hollow, thin-walled, biocompatible polymer material, shown in Figure 3, has extremely thin slits 2 that are laser cut into flexible catheter 4 at regular intervals near closed distal end 6. The slits, which are

referred to as pressure response outlets ("PRO"), are tight enough that infusate will not escape unless the fluid pressure within the catheter reaches a critical point and causes the slits to distend simultaneously and thus open temporarily. The catheter can also contain exterior radiopaque markers 8 to assist in the positioning of the device at the site of the thrombus.

Ideally, such "PRO" infusion catheters are used in conjunction with an automated, piston-driven, pulsed infusion device (not shown) that is capable of delivering low volume regulated pulses of drug infusion into entry port 10 in mating ring 12 affixed at proximal end 14 of catheter 4. When a pulse is delivered, the pressure within the catheter rises momentarily. In response, the pressure response outlets (slits 2) open momentarily and allow the infusate (e.g., pharmaceutical solution of fibrinolytic metalloproteinase) to escape. The theoretic advantage of pulsed delivery of infusate and a PRO-type catheter is that infusate is delivered uniformly through the slits along the entire length of the catheter, whereas infusate delivered through the "side hole" catheter (Figure 2) follows a path of least resistance and flows out the proximal side holes in a non-uniform manner as mentioned.

A pig model of four-day old carotid thrombosis was utilized to assess performance of the above-mentioned two catheter types, using fixed dosages of 30 mg of NAT (approximately 0.4 mg/kg in a 75 kg pig). The results are summarized in Table 1.

TABLE 1

Comparison of Angiographic Flow Scores Obtained with NAT Using Side Hole and PRO Infusion Catheters

				ANGIOGRAPHIC FLOW SCORE				
DRUG	DOSE	CATH	DELIV TIME (delivery)	0	30m	1hr	2hr	4hr
NAT n=5	30 mg	CM	60 min (infusion)	0.0 ± 0.0	1.2 ± 0.4	1.6 ± 0.5	1.8 ± 0.4	2.0 ± 0.7
NAT n=4	30 mg	PS	60 min (0.1mL pulse)	0.0 ± 0.0	1.5 ± 0.6	1.5 ± 0.6	2.3 ± 0.5	2.5 ± 0.6

CM: Cragg-McNamara™ valved infusion "side hole" type catheter (Micro Therapeutics, Inc., San Clemente, California). PS: "pulse-spray" delivery as defined by use of pressure response outlet (PRO) catheter (Uni*Fuse Catheter™, AngioDynamics, Inc., Queensbury, New York) used in conjunction with automated, pulsed infusion device (PULSE*SPRAY INJECTOR Model PSI-1, AngioDynamics, Inc., Queensbury, New York).

As shown in Table 1, angiographic flow scores in the group treated with the pulse-spray modality showed slightly higher initial flow scores at the thirty-minute angiogram which appeared to persist to the four-hour timepoint. Although statistical differences were not obtained, the angiographic results have generally been judged to be superior. Therefore, a PRO-type catheter in conjunction with pulse-spray delivery is the preferred mode of delivery for a fibrinolytic metalloproteinase in accordance with this invention.

EXAMPLE 3

Assessment of Drug Delivery Time

Acute peripheral arterial occlusion is typically treated with plasminogen activators, such as urokinase, delivered as an infusion which is often twenty-four hours in duration, and occasionally as long as forty-eight hours. The lengthy infusion is presumed to maintain a low level of plasmin generation over a prolonged period of time in order to effectively dissolve the occlusive thrombus. As both NAT and fibrolase are fibrinolytic metalloproteinases, such prolonged infusions may not be necessary. To assess whether the delivery rate affects angiographic clot lysis, a fixed 30 mg dose of NAT (roughly 0.4 mg/kg in a 75 kg pig) was delivered using PRO catheters and the pulse spray device. Using 0.1 mL pulse volumes, a 5 mg/mL NAT solution was delivered over six minutes (ten pulses per minute) or over sixty minutes (one pulse per minute). The results are shown in Table 2.

TABLE 2

Comparison of Drug Delivery Times for NAT Delivered by PRO Catheter and Pulse-Spray Injector

				ANGIOGRAPHIC FLOW SCORE				
DRUG	DOSE	CATH	DELIV TIME (pulse volume)	0	30m	1hr	2hr	4hr
NAT n=3	30 mg	PS	6 min (0.1mL)	0.0 ± 0.0	2.7 ± 0.6	2.7 ± 0.6	2.7 ± 0.6	2.7 ± 0.6
NAT n=3	30 mg	PS	60 min (0.1mL)	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 1.0	1.3 ± 1.2	1.7 ± 0.6

As shown in Table 2, the delivery of 30 mg NAT over six minutes resulted in a mean flow score of 2.7

in the three animals at the thirty-minute angiogram, which persisted out to the four-hour timepoint. In contrast, the delivery of 30 mg of NAT by pulsed infusion over sixty minutes was far less impressive. Although these data have not been statistically compared, the results appear to favor delivery of NAT as a more rapid pulse regimen.

EXAMPLE 4

Optimization of Pulse Volume

The pulse-spray infusion device is programmable for delivering pulse volumes of 0.1 to 0.5 mL per pulse. To determine if the pulse volume had any effect on the angiographic outcomes in the pig model, pulse volumes of 0.2 mL were compared to pulse volumes of 0.4 mL. NAT was delivered at a fixed dose of 10 mg (equivalent to 0.15 mg/kg in a 75 kg pig). The results are shown in Table 3.

TABLE 3

Comparison of Angiographic Results Using NAT Delivered in Pulse Volumes of 0.2 mL or 0.4 mL

				ANGIOGRAPHIC FLOW SCORE				
DRUG	DOSE	CATH	DELIV TIME (pulse volume)	0	30m	1hr	2hr	4hr
NAT n=7	10 mg 2.5 mg/mL	PS	5 min (0.2mL q15sec)	0.0 ± 0.0	1.0 ± 0.6	1.1 ± 0.7	1.6 ± 1.0	1.4 ± 1.1
NAT n=6	10 mg 2.5 mg/mL	PS	5 min (0.4mL q30sec)	0.0 ± 0.0	1.2 ± 0.4	1.0 ± 0.6	0.8 ± 0.8	1.0 ± 0.6

As shown in Table 3, at thirty minutes the mean angiographic score was slightly higher in the 0.4 mL pulse volume group. However, at the four-hour time point, the group mean was slightly higher in the 0.2 mL pulse volume. As such, no conclusion can be drawn from these studies with regard to one pulse volume being superior to another.

The results in preceding text and tables suggest that virtually all of these NAT treatment regimens are effective in treating peripheral artery occlusions with the method of delivery of this invention, and that these results are at least comparable to treatment with plasminogen activators, such as urokinase, which is the current treatment of choice with thrombolytic agents.

The results demonstrate that the PRO catheter with pulse-spray delivery appears to provide superior angiographic results. Given the body weight of animals in these studies (70-100 kg), the fixed dosage of 30 mg is roughly equivalent to 0.3-0.4 mg/kg on a weight-adjusted basis.

Lowering the fixed dosage NAT to 10 mg resulted in a reduction of group mean angiographic scores at four hours and in some animals, patency was not achieved. This indicates that a dose of 10 mg of NAT appears to be a threshold dose for biologic activity in this model. Given the body weight of animals in these studies (70-100 kg), the fixed dosage of 10 mg is roughly equivalent to 0.1-0.15 mg/kg on a weight-adjusted basis. Varying the pulse volume from 0.2 mL to 0.4 mL did not appear to profoundly impact the angiographic patency scores.

EXAMPLE 5

Establishment of Safe, Well Tolerated, Biologically Effective Dose Range in Humans

No satisfactory literature exists on the serum concentration or biochemical activity of α_2-macroglobulin in elderly patients with peripheral vascular disease (PVD). As α_2-macroglobulin concentrations are a key determinant of the safety and likely to be related the tolerabilty of fibrinolytic metalloproteinases *in vivo*, a cross-sectional epidemiological study was conducted in patients with PVD to evaluate serum α_2-macroglobulin concentration and the fibrinolytic metalloproteinase binding capacity (using NAT as the test agent).

Two hundred and sixteen patients were enrolled at two centers (Cleveland Clinic Foundation, Cleveland, OH, and Rochester General Hospital, Rochester, NY). Demographic information and other patient characteristics were collected and serum was obtained for measuring α_2-macroglobulin, the NAT binding capacity (by titration of individual patient serum samples using an HPLC assay that detects unbound NAT) and other serum chemistry parameters. The primary endpoint was the determination of the relationship between the serum concentration of α_2-macroglobulin and the amount of NAT (in micrograms per milliliter of serum) that could be neutralized *in vitro* (NAT binding capacity).

A comparison of patient characteristics in this study with those of the two largest published studies of thrombolysis in PAO (i.e., the STILE and TOPAS studies) indicated that the patient population in this study was representative of previous studies of thrombolysis in PAO; For further details on the previous studies, see Annals of Surgery, Volume 220, pages 251-266 (1994) and Ouriel et al., New England Journal of Medicine, Volume 338, pages 1105-1111 (1998), respectively.

The estimated maximum dose (EMD) for NAT was calculated for each patient using the NAT binding capacity and an estimate of each patient's plasma volume. The study results predict that the average patient could receive a dosage of 1.7 mg/kg (delivered either locally or systemically) without exceeding the capacity of α_2-macroglobulin to bind and neutralize NAT. The results from the study are summarized in Figure 4..

In Figure 4, the estimated maximum dose of NAT was calculated for each of the 216 subjects in the study. The results for the study are depicted as a histogram, above, where a bell-shaped distribution can be observed by visual inspection. The average patient in this study is predicted to be capable of tolerating 1.7 mg/kg of NAT (the peak of the bell-shaped distribution). Dosages administered in animal studies are shown for reference (right hand side) and can be seen to be in excess of the estimated maximum dose of NAT for 99% of the study population.

Thus, the prescribed range of 0.025 to 1.7 mg/kg for the invention represents a rational estimate of the dose patients can safely receive (based on plasma volume and NAT binding capacity for α_2-macroglobulin) without the appearance of free NAT in the circulation.

In conclusion, the results from the exemplified pharmacology studies, Examples 1-4, above, indicate the biological effectiveness of a fibrinolytic metalloproteinase as a clot lysing agent in an animal model of thrombosis where the thrombus is comparable in size and age to that frequently encountered in peripheral arterial occlusion in humans. The dosages identified in the animal models were obtained without regard for or assessment of the potential toxicities in the animal. The effective dosages in rabbits and dogs (3.7 and 4.0 mg/kg, respectively) as described by Ahmed et al. and Markland et al. (above) might enable a veterinary use for fibrinolytic metalloproteinases. However, when considered in the presence of human data, the administration of doses of 3.7 and 4.0 mg/kg would have overdosed 99 percent of the study population. Therefore, the published animal studies do not enable the therapeutic use of fibrinolytic metalloproteinases in humans in a manner that is safe as well as biologically effective. The data presented in Example 5, on the other hand, do enable such use in humans.

CLAIMS

1. Use of a fibrinolytic metalloproteinase selected from the group consisting of fibrolase and Novel Acting Thrombolytic (NAT) in the manufacture of a composition for lysing a blood clot in, on, or attached to an indwelling vascular access device to restore patency to the indwelling vascular access device.

2. Use of a fibrinolytic metalloproteinase selected from the group consisting of fibrolase and Novel Acting Thrombolytic (NAT) in the manufacture of a composition for lysing a blood clot in, on, or attached to an indwelling vascular access device to restore function to the indwelling vascular access device.

3. The use of claim 1 or 2, wherein the fibrinolytic metalloproteinase is present in the composition in a concentration ranging from 5.0 to 4.0 mg/ml.

4. The use of claim 1 or 2, wherein the fibrinolytic metalloproteinase is present in the composition in an amount ranging from 0.5 to 4.0 mg.

5. The use of claim 4, wherein the fibrinolytic metalloproteinase is present in the composition in an amount selected from the group consisting of 0.5 mg, 1.0 mg, 2.0 mg, 3.0 mg, and 4.0 mg.

6. The use of claim 1 or 2, wherein said vascular access device is a catheter.

7. The use of claim 1 or 2, wherein said vascular access device is a shunt.

8. The use of claim 1 or 2, wherein said vascular access device is an access graft.

9. The use of claim 1 or 2, wherein said vascular access device is a needle.

10. The use of claim 1 or 2, wherein said vascular access device is used to introduce a liquid composition into a human artery or vein.

11. The use of claim 1 or 2, wherein said vascular access device is used to remove blood from a human artery or vein.

12. The use of claim 1 or 2, wherein said vascular access device is used in connection with a hemodialysis procedure.

13. The use of claim 1 or 2, wherein said vascular access device is used in connection with a blood transfusion procedure.

14. The use of claim 1 or 2, wherein said vascular access device is used in connection with chemotherapy.

15. The use of claim 1 or 2, wherein said vascular access device is used in connection with blood removal.

16. The use of claim 1 or 2, wherein the blood clot is located on an interior surface of the dwelling vascular access device.

17. The use of claim 1 or 2, wherein the blood clot is located on an exterior surface of the dwelling vascular access device.

18. The use of claim 1 or 2, wherein the blood clot is attached to the indwelling vascular access device.

19. The use of claim 1 or 2, wherein said indwelling vascular access device is located in an artery.

20. The use of claim 1 or 2, wherein said indwelling vascular access device is located in a vein.

21. The use of claim 1 or 2, wherein said indwelling vascular access device comprises a side hole catheter device.

22. The use of claim 1 or 2, wherein said indwelling vascular access device comprises a pressure response outlet (PRO) catheter delivery device.

23. The use of claim 1 or 2, wherein the fibrinolytic metalloproteinase is administered by bolus.

24. The use of any one of claims 1-23, wherein the fibrinolytic metalloproteinase comprises NAT.

25. The use of any one of claims 1-23, wherein the fibrinolytic metalloproteinase comprises fibrolase.

SEQUENCE LISTING

<110> Toombs, Christopher F.

<120> METHOD FOR TREATMENT OF INDWELLING CATHETER OCCLUSION US FIBRINOLYTIC METALLOPROTEINASES

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gacttcgacg gtgatactgt tggtctggct tacgttggtg gcatgtgtca actgaaacat 360

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cacgaactgg gtcataacct gggtatgaac cacgatggca accagtgtca ctgcggtgca 480

aactcctgtg ttatggctgc tatgctgtcc gatcaaccat ccaaactgtt ctccgactgc 540

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ccg 603

<210> 3
<211> 203
<212> PRT
<213> Agkistrodon contortrix

<220>
<221> MISC_FEATURE
<223> Native fibrolase of Agkistrodon Contortrix

<400> 3

Gln Gln Arg Phe Pro Gln Arg Tyr Val Gln Leu Val Ile Val Ala Asp
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His Arg Met Asn Thr Lys Tyr Asn Gly Asp Ser Asp Lys Ile Arg Gln
20 25 30

Trp Val His Gln Ile Val Asn Thr Ile Asn Glu Ile Tyr Arg Pro Leu
35 40 45

Asn Ile Gln Phe Thr Leu Val Gly Leu Glu Ile Trp Ser Asn Gln Asp
50 55 60

Leu Ile Thr Val Thr Ser Val Ser His Asp Thr Leu Ala Ser Phe Gly

65 70 75 80

Asn Trp Arg Glu Thr Asp Leu Leu Arg Arg Gln Arg His Asp Asn Ala
85 90 95

Gln Leu Leu Thr Ala Ile Asp Phe Asp Gly Asp Thr Val Gly Leu Ala
100 105 110

Tyr Val Gly Gly Met Cys Gln Leu Lys His Ser Thr Gly Val Ile Gln
115 120 125

Asp His Ser Ala Ile Asn Leu Leu Val Ala Leu Thr Met Ala His Glu
130 135 140

Leu Gly His Asn Leu Gly Met Asn His Asp Gly Asn Gln Cys His Cys
145 150 155 160

Gly Ala Asn Ser Cys Val Met Ala Ala Met Leu Ser Asp Gln Pro Ser
165 170 175

Lys Leu Phe Ser Asp Cys Ser Lys Lys Asp Tyr Gln Thr Phe Leu Thr
180 185 190

Val Asn Asn Pro Gln Cys Ile Leu Asn Lys Pro
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<210> 4
<211> 609
<212> DNA
<213> Agkistrodon contortrix

<220>
<221> misc_feature
<223> Encodes native fibrolase of Agkistrodon Contortrix

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attaacgaaa tctacagacc actgaacatc caattcactt tggttggttt ggaaatctgg 180

tccaaccaag atttgatcac cgttacttct gtatcccacg acactctggc atccttcggt 240

aactggcgtg aaaccgacct gctgcgtcgc caacgtcatg ataacgctca actgctgacc 300

gctatcgact tcgacggtga tactgttggt ctggcttacg ttggtggcat gtgtcaactg 360

aaacattcta ctggtgttat ccaggaccac tccgctatta acctgctggt tgctctgacc 420

atggcacacg aactgggtca taacctgggt atgaaccacg atggcaacca gtgtcactgc 480

ggtgcaaact cctgtgttat ggctgctatg ctgtccgatc aaccatccaa actgttctcc 540

gactgctcta agaaagacta ccagaccttc ctgaccgtta acaacccgca gtgtatcctg 600

aacaaaccg 609

<210> 5
<211> 1392
<212> DNA
<213> Agkistrodon contortrix

<220>
<221> misc_feature
<223> Native profibrolase of Agkistrodon Contortrix

<400> 5
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ccagtcaaca ctacaacaga agatgaaacg gcacaaattc cggctgaagc tgtcatcggt 120

tactcagatt tagaagggga tttcgatgtt gctgttttgc cattttccaa cagcacaaat 180

aacgggttat tgtttataaa tactactatt gccagcattg ctgctaaaga agaagggta 240

tctctcgaga aaagagaggc tgaagcttct tctattatct tggaatctgg taacgttaac 300

gattacgaag ttgtttatcc aagaaaggtc actccagttc ctaggggtgc tgttcaacca 360

aagtacgaag atgccatgca atacgaattc aaggttaaca gtgaaccagt tgtcttgcac 420

ttggaaaaaa acaaaggttt gttctctgaa gattactctg aaactcatta ctccccagat 480

ggtagagaaa ttactactta cccattgggt gaagatcact gttactacca tggtagaatc 540

gaaaacgatg ctgactccac tgcttctatc tctgcttgta acggtttgaa gggtcatttc 600

aagttgcaag gtgaaatgta cttgattgaa ccattggaat tgtccgactc tgaagcccat 660

gctgtctaca agtacgaaaa cgtcgaaaag gaagatgaag ccccaaagat gtgtggtgtt 720

acccaaaact gggaatcata tgaaccaatc aagaaggcct tccaattaaa cttgactaag 780

agacaacaaa gattcccaca aagatacgta cagctggtta tcgttgctga ccaccgtatg 840

aacactaaat acaacggtga ctctgacaaa atccgtcaat gggtgcacca aatcgtcaac 900

accattaacg aaatctacag accactgaac atccaattca ctttggttgg tttggaaatc 960

tggtccaacc aagatttgat caccgttact tctgtatccc acgacactct ggcatccttc 1020

ggtaactggc gtgaaaccga cctgctgcgt cgccaacgtc atgataacgc tcaactgctg 1080

accgctatcg acttcgacgg tgatactgtt ggtctggctt acgttggtgg catgtgtcaa 1140

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tgcggtgcaa actcctgtgt tatggctgct atgctgtccg atcaaccatc caaactgttc 1320

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ctgaacaaac cg 1392

<210> 6
<211> 1386
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: proNAT (analog of profibrolas
e of Agkistrodon Contortrix

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ccagtcaaca ctacaacaga agatgaaacg gcacaaattc cggctgaagc tgtcatcggt 120

tactcagatt tagaagggga tttcgatgtt gctgttttgc cattttccaa cagcacaaat 180

aacgggttat tgtttataaa tactactatt gccagcattg ctgctaaaga agaaggggta 240

tctctcgaga aaagagaggc tgaagcttct tctattatct tggaatctgg taacgttaac 300

gattacgaag ttgtttatcc aagaaaggtc actccagttc ctaggggtgc tgttcaacca 360

aagtacgaag atgccatgca atacgaattc aaggttaaca gtgaaccagt tgtcttgcac 420

ttggaaaaaa acaaaggttt gttctctgaa gattactctg aaactcatta ctccccagat 480

ggtagagaaa ttactactta cccattgggt gaagatcact gttactacca tggtagaatc 540

gaaaacgatg ctgactccac tgcttctatc tctgcttgta acggtttgaa gggtcatttc 600

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gctgtctaca agtacgaaaa cgtcgaaaag gaagatgaag ccccaaagat gtgtggtgtt 720

acccaaaact gggaatcata tgaaccaatc aagaaggcct tccaattaaa cttgactaag 780

agatctttcc cacaaagata cgtacagctg gttatcgttg ctgaccaccg tatgaacact 840

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aacgaaatct acagaccact gaacatccaa ttcactttgg ttggtttgga aatctggtcc 960

aaccaagatt tgatcaccgt tacttctgta tcccacgaca ctctggcatc cttcggtaac 1020

tggcgtgaaa ccgacctgct gcgtcgccaa cgtcatgata acgctcaact gctgaccgct 1080

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cattctactg gtgttatcca ggaccactcc gctattaacc tgctggttgc tctgaccatg 1200

gcacacgaac tgggtcataa cctgggtatg aaccacgatg gcaaccagtg tcactgcggt 1260

gcaaactcct gtgttatggc tgctatgctg tccgatcaac catccaaact gttctccgac 1320

tgctctaaga aagactacca gaccttcctg accgttaaca acccgcagtg tatcctgaac 1380

aaaccg 1386