Thrombolytic Effects of Recombinant Fibrolase or APSAC in a Canine Model of Carotid Artery Thrombosis

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Background  Thrombolytic agents used clinically rely on the activation of plasminogen to plasmin. Plasmin possesses multiple actions including increasing thrombin activity and activation of platelets. Thus, after successful thrombolytic therapy, arterial hyperactivity and reocclusion may be the result of a predominant plasmin-induced thrombogenic action at the site of the residual thrombus. Fibrolase, a direct-acting fibrinolytic enzyme from southern copperhead snake venom, induces rapid clot lysis in vitro. Fibrolase does not rely on plasminogen activation or any other bloodborne components for activity and is not inhibited by any of the rapidly acting serine proteinase inhibitors in blood.  

Methods and Results  We investigated the efficacy of fibrolase to lyse an occlusive thrombus formed in the carotid artery of the anesthetized dog. Electrolytic injury was initiated in both the right and left carotid arteries. Thirty minutes after both arteries were occluded, each vessel was infused with either fibrolase (4 mg/kg over 5 minutes) or physiological saline (over 5 minutes). In two separate groups of dogs, anisoylated plasminogen streptokinase activator complex (APSAC) (0.1 U/kg) was infused into the occluded vessel. In the artery infused with fibrolase, five of five dogs exhibited patency within 6±1 minutes of the infusion (P<0.05 versus vehicle-treated artery; Fisher’s exact test). In the contralateral carotid artery that received vehicle, the occlusion was maintained throughout the experimental protocol. APSAC alone lysed the thrombus in each vessel within 17±3 minutes. Five minutes after the end of fibrolase administration and in one of the groups administered APSAC, a glycoprotein (GP)IIb/IIIa antibody, 7E3 (0.8 mg/kg IV), was administered to prevent reocclusion of the patent artery. After 7E3 administration, the vessel treated with fibrolase remained patent in four of five dogs, and six of six APSAC-treated vessels were patent for the remainder of the observation period (2 hours).

Conclusions  These studies demonstrate that local administration of fibrolase lysed a carotid arterial thrombus rapidly without excessive hemorrhage or hemodynamic compromise. The enzyme in combination with antiplatelet therapy (7E3) offers a unique mechanism for clot dissolution and may prove useful as a clinically efficacious alternative to presently used thrombolytic agents or may act in a synergistic manner with plasminogen activators. (Circulation. 1994;90:2448-2456.)

Key Words  • thrombolysis  • antibodies  • plasminogen

Despite the clinical efficacy of currently used thrombolytic agents, excessive bleeding as well as the failure to achieve rapid and complete clot lysis have provided the stimulus to search for a more optimal means of achieving thrombolysis. Thrombolytic agents in current clinical use activate plasminogen, leading to the formation of plasmin, a serine proteinase.1 Plasmin, in addition to degrading fibrin and facilitating clot lysis, may actually lead to platelet activation and promote a thrombogenic state at the site of the original occlusive lesion.2-6 Thrombin activity, as determined by the increase in the plasma concentration of fibrinopeptide A, a peptide cleaved from fibrinogen by thrombin, increases in patients given recombinant tissue-type plasminogen activator (rt-PA) or streptokinase (SK).7,8 The plasmin-induced increase in thrombus-associated thrombin leads to platelet aggregation at the site of the residual thrombus and may, in concert with other factors, contribute to coronary artery reocclusion after successful thrombolysis.2,4 Platelet activation and aggregation in the region of a residual thrombus may be important contributing factors to thrombus formation and vessel reocclusion.10 The inherent plasmin-related deficiencies of clinically available thrombolytic agents provide the impetus to examine alternative approaches and develop improved lytic agents with the goals of enhancing the incidence of recanalization, preventing reocclusion, and reducing the frequency of bleeding complications.11

In view of the potential limitations of plasminogen activator–based thrombolytic agents, we have chosen to investigate the thrombolytic activity of a fibrinolytic enzyme purified from the southern copperhead snake (Agkistrodon contortrix contortrix) venom.12,13 The enzyme, identified as fibrolase, is a zinc-containing metalloproteinase (1 mol of zinc per mole of enzyme) with a molecular mass of 23 kDa.13 Fibrolase possesses direct-acting fibrinolytic activity and does not activate plasminogen or protein C nor does it require any bloodborne components for activity.14 The enzyme degrades the α- and β-chains of fibrin and fibrinogen. There is little degradation of the γ-chain or the γ-γ dimer. Mecha-
nisms that promote resistance of thrombi to lysis by plasminogen activator–based thrombolytic agents, including factor XIII-mediated incorporation of α2-antiplasmin into fibrin\textsuperscript{15,16} or inhibition by plasminogen activator inhibitors (eg, PAI-1).\textsuperscript{17} would not apply with fibrolase, which is a metalloproteinase and is not inhibited by serine proteinase inhibitors.

Recently, southern copperhead snake venom gland mRNA has been isolated and full-length fibrolase cDNA prepared. Secretion of recombinant fibrolase (r-fibrolase) from yeast has been achieved by a plasmid-mediated expression system.\textsuperscript{18} r-Fibrolase yields of 30 to 60 mg/L are obtained using a proteinase-deficient yeast strain in a defined medium containing zinc. The recombinant enzyme has been purified by a four-step procedure and shown to be identical to the natural enzyme with respect to physicochemical properties and biological activity.\textsuperscript{18}

The primary intent of this investigation was to determine if r-fibrolase, a zinc-containing metalloproteinase, could achieve thrombolysis in an experimental animal model in which a platelet-rich, occlusive arterial thrombus develops in response to a deep arterial wall injury. We examined the thrombolytic activity of r-fibrolase and anisoylated plasminogen streptokinase activator complex (APSAC) using an experimental model of arterial thrombosis capable of exhibiting thrombolysis and reocclusion. The model used in this study is similar in principle to previously established coronary models\textsuperscript{19} but utilizes a carotid artery to minimize experimental loss due to ventricular fibrillation. In the present studies, we report that the combination of fibrolase and the 7E3 F(ab')2 monoclonal glycoprotein (GP)\textsubscript{IIb/IIIa} receptor antibody produces rapid and sustained thrombolysis in the canine carotid arterial thrombosis model system.

Methods

Animal Investigation

The studies conform to the Position of the American Heart Association on Research Animal Use adopted November 11, 1984. The procedures were in accordance with the guidelines of the University of Michigan (Ann Arbor) University Committee on the Use and Care of Animals. Veterinary care was provided by the University of Michigan Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Association of Accreditation of Laboratory Animal Care, and the animal care and use program conforms to the standards in "The Guide for Care and Use of Laboratory Animals," Department of Health, Education, and Welfare Publication No. NIH 78-23.

Instrumentation

Eighteen male mongrel dogs (weight, 7 to 20 kg) were selected for study. Each dog was anesthetized with sodium pentobarbital (30 mg/kg IV), intubated, and ventilated with room air and positive pressure at a stroke volume of 30 mL/kg and a frequency of 12 breaths per minute (Harvard Apparatus). Both common carotid arteries and the right internal jugular vein were exposed. A catheter was inserted into the jugular vein for blood sampling and administration of the test drug. Arterial blood pressure was monitored from the cannulated femoral artery with the use of a blood pressure transducer (Gould Inc, Cardiovascular Products). Heart rate was recorded throughout the experimental protocol from the standard limb lead II of the ECG. A Doppler flow probe (model 100, Triton Technology) was placed on each common carotid artery proximal to both the point of insertion of the intraarterial electrode and the mechanical constrictor. The mechanical constrictor is constructed of stainless steel in a C-shape with a Teflon screw (2-mm diameter) that is adjusted to control vessel circumference and produce a regional stenosis. The constrictor is adjusted until the pulsatile flow pattern is reduced by 25% to 30% without altering mean flow. Blood flow velocity in each carotid artery was monitored continuously. Recordings of blood pressure, limb lead II ECG, as well as pulsatile flow velocity of each carotid artery was recorded on a model 7 polygraph recorder (Grass Instruments).

Electrolytic injury to the intimal surface of each carotid artery was accomplished with the use of an intravascular electrode composed of a Teflon-insulated, silver-coated copper wire. Penetration of the vessel wall by the electrode is facilitated by attaching the tip of a 25-gauge hypodermic needle to the uninsulated portion of the wire. Each intraarterial electrode is connected to the anode (+) of a dual-channel stimulator (Grass S88 stimulator and a Grass Constant Current Unit, model CCU1A, Grass Instrument Co). The cathode is attached to a distant subcutaneous site. The current delivered to each vessel is monitored continuously on separate ammeters and maintained at 300 μA. The anodal electrode is positioned to have the uninsulated portion of the electrode in contact with the endothelial surface of the vessel. Proper positioning of the electrodes in each of the carotid arteries is confirmed by visual inspection at the end of each experiment (see Fig 1).

Experimental Protocol Group

The anodal current is applied to the intimal surface of the carotid artery for a maximum period of 4 hours or is terminated 30 minutes after blood flow in each vessel remains stable at zero flow velocity to verify having achieved formation of a stable occlusive thrombus. Three experimental groups of six animals per group were studied. Group A consisted of animals in which the right carotid artery underwent electrolytic injury and thrombosis followed by the intra-arterial administration of APSAC 0.1 U/kg immediately proximal to the occlusive lesion. The intra-arterial administration of each compound was through a curved, 26-gauge needle attached to PE50 tubing.
Group B animals were similar to the previous group, but in addition were treated with a single intravenous dose of 0.8 mg/kg of the GPIIb/IIIa receptor antagonist, monoclonal antibody 7E3 F(ab')2. Group C animals had both carotid arteries subjected to electrolytic injury, leading to occlusive thrombus formation. Both arteries are instrumented in an identical manner, and vessel wall injury is induced simultaneously in each carotid artery. Fibrolase (4 mg/kg in a volume of 3 mL) was infused over 5 minutes proximal to the thrombus in the left carotid artery only. Sodium chloride solution (0.9%, identical rate and volume as the fibrolase infusion) was infused simultaneously, proximal to the thrombus in the right carotid artery. The opposing carotid artery was used as a control vessel to allow each dog to serve as its own control as well as minimizing the number of dogs used to complete the study. If lysis and reperfusion were achieved, 0.8 mg/kg of the GPIIb/IIIa receptor antagonist, monoclonal antibody 7E3 F(ab')2, was administered intravenously as in the previously described group B. We have demonstrated in the past that the monoclonal 7E3 antibody is capable of attenuating reocclusion after thrombolysis in a similar model system.

Reperfusion is defined as the restoration of carotid artery blood flow velocity to 20% of baseline values. Patency is defined as measurable carotid artery blood flow velocity. Blood pressure, heart rate, and carotid artery flow velocity were monitored for 2 hours after achieving successful thrombolysis.

At the conclusion of the study protocol, each vessel segment was ligated and removed without disturbing the intravascular thrombus. The vessel segment was opened along its length, and the intact thrombus mass was lifted off the intimal surface of the vessel. The weight of the thrombus was determined with an analytical balance. After respective vessel segments had been obtained, each dog was euthanized, and a postmortem examination was performed. The chest was opened, and the pericardium was exposed for evidence of excessive blood infiltration. The appearance of the heart and surrounding pericardium appeared normal in each dog. The intestines, liver, and kidneys were examined and found to be normal in appearance. We were unable to find evidence of hemorrhaging at any site in any of the dogs that received r-fibrolase.

Hematologic Measurements

Venous blood samples (20 mL) for evaluation of platelet aggregation were withdrawn from the jugular cannula into a plastic syringe containing 3.2% sodium citrate as the anticoagulant (1:10 vol/vol, citrate/blood) at baseline, 5 minutes after thrombolysis by APSAC or fibrolase, 30 minutes after completion of the infusion of 7E3, and 2 hours after infusion of 7E3. Concentrations of red blood cells, white blood cells, hemoglobin, platelets, and the hematocrit were determined with an H-10 cell counter (Texas International Laboratories, Inc). Platelet-rich plasma (PRP), the supernatant present after centrifugation of anticoagulated whole blood at 1000 rpm for 5 minutes (140g), is diluted with platelet-poor plasma (PPP) to achieve a platelet count of 200,000/mL. PPP is prepared after the PRP is removed by centrifuging the remaining blood at 3000 rpm (2000g) for 10 minutes and discarding the bottom cell layer. Ex vivo platelet aggregation is determined by established turbidometric methods with a four-channel aggregometer (BioData-PAP-4, Bio Data Corp) by recording the increase in light transmission through a stirred suspension of PRP maintained at 37°C. Aggregation is induced with arachidonic acid (0.65 and 0.325 mmol/L) or ADP (20 and 5 μmol/L). A subaggregatory dose of epinephrine (550 mmol/L) is used to prime the platelets before addition of the respective agonists. Values are expressed as percentage of aggregation, which represents the percentage of light transmission standardized to PRP and PPP samples yielding 0% and 100% light transmission, respectively.

To assess the anticoagulation status of the animals, activated partial thromboplastin time (aPTT) was determined using a Hemochron (Technidyne) and the reagents supplied by the manufacturer. Citrated venous blood was collected for these determinations.

Inclusion Criteria

For inclusion of animals into the protocol, several established criteria were required to be met: (1) a baseline circulating platelet count of not less than 200,000/mL, (2) definitive aggregation of predrug platelets in response to arachidonic acid and ADP, (3) thrombotic occlusion of either vessel within 4 hours of vessel wall electrolytic injury, and (4) absence of heart worms upon final necropsy examination.

r-Fibrolase Preparation

r-Fibrolase was produced by a yeast expression system by investigators at Chiron Corporation. The recombinant enzyme was purified from yeast broth by a four-step chromatographic procedure including Q-Sepharose anion exchange open-column chromatography, phenyl Sepharose hydrophobic interaction open-column chromatography, Sephacryl S100 molecular sieve open-column chromatography, and CM300 cation exchange high-performance liquid chromatography.18 The purified enzyme was dialyzed against 10 mmol/L Tris-HCl, 100 mmol/L NaCl (pH 7.4), then concentrated to approximately 20 mg/mL and stored at −80°C.

Statistical Analysis

Data are expressed as mean±SEM. A repeated-measures ANOVA was performed to test the interaction between time and treatment and the overall effects of groups and time. Platelet aggregation, aPTT, and hematologic measures over time were analyzed with one-way ANOVA (repeated measures). The Student’s t test for paired comparison was used to test differences between rates of occlusion in the left versus right carotid arteries in the same animal. Nominal data concerning incidence of lysis were analyzed with a Fisher’s exact test. Statistical calculations were done using a Macintosh SE/30 (Apple Computer) and STATVIEW software (Abacus Concepts, Inc).

Results

A total of 18 dogs weighing 12±1 kg were entered into the study and allocated among three groups. One dog assigned to group C was excluded because it did not meet three of the four inclusion criteria described above. Group A animals (15±2 kg, n=6) received APSAC (0.1 U/kg). Group B animals (13±1 kg, n=6) received APSAC (0.1 U/kg) plus 7E3 F(ab')2 monoclonal antibody (0.8 mg/kg IV) 5 minutes after achieving clot lysis. Group C animals (9±1 kg, n=5) were administered r-fibrolase (4.0 mg/kg) plus 7E3 F(ab')2 monoclonal antibody (0.8 mg/kg IV) 5 minutes after thrombolysis.

Group A: APSAC-Induced Thrombolysis

The six animals in group A developed occlusive arterial thrombi in 133±12 minutes. The infusion of APSAC alone immediately proximal to the occlusive lesion lysed the thrombus in each of the six animals within a mean time of 17±3 minutes (Fig 2). Despite the efficacy of APSAC in achieving thrombolysis, reocclusion of the carotid artery occurred in all animals within an additional 40±5 minutes (Fig 3A). Blood flow velocity throughout the experimental protocol is summarized in Fig 4A. The mean weight of the thrombi removed from the occluded carotid arteries was 62±9 mg.
Group B: APSAC-Induced Thrombolysis Plus 7E3 F(ab')2 Monoclonal Antibody

The mean time to carotid artery occlusion for the six animals of group B was 108±13 minutes. The occlusive carotid artery thrombi in group B animals treated with APSAC lysed within 26±8 minutes (Fig 2). After clot lysis was achieved, 7E3 F(ab')2 monoclonal antibody was administered and the carotid arteries remained patent in each of the six animals for the remainder of the experimental protocol (Fig 3B). Blood flow velocity throughout the experimental protocol is summarized in Fig 4A. The carotid artery thrombi removed at the end of the study had a mean weight of 30±8 mg.

Group C: r-Fibrolase-Induced Thrombolysis Plus 7E3 F(ab')2 Monoclonal Antibody

Group C animals were subjected sequentially to thrombus formation in the right and left carotid arteries. The administration of 0.9% sodium chloride (3.0 mL over 5 minutes) proximal to the thrombus in the right carotid artery failed to achieve clot lysis in each of the five animals. The administration of r-fibrolase proximal to the thrombus in the left carotid artery achieved thrombolysis in all animals, within a mean time of 6±1 minutes (Fig 2). The subsequent administration of 7E3 F(ab')2 monoclonal antibody maintained left carotid artery patency in four of the five animals so treated. One animal (No. 3) rethrombosed despite the presence of the 7E3 F(ab')2 monoclonal antibody (Fig 3C). The results for group C are summarized graphically in Fig 4B, which compares the blood flow velocity in the left carotid arteries (infused with r-fibrolase plus 7E3) ver-

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Fig 2. Scattergrams representing time to occlusion (upper), time to lysis (middle), and total reperfusion time (lower) for each of the treatment groups. Each symbol represents an individual animal. APSAC indicates anisoylated plasminogen streptokinase activator complex.

Fig 3. Composite graphic representation of carotid artery blood flow in each of three study groups. The organization of each panel is as follows: Each horizontal bar represents one experimental animal in which electrolytic injury is induced in the carotid artery for the purpose of inducing an occlusive arterial thrombus. The dark portion of each horizontal bar indicates the period during which the Doppler flow probe failed to record blood flow velocity in the carotid artery due to the presence of an occlusive thrombus. The light segment in each bar represents the interval during which blood flow, as determined by the presence of a Doppler signal, was present in the carotid artery. Group A consisted of 6 animals that underwent unilateral carotid artery thrombosis followed by administration of anisoylated plasminogen streptokinase activator complex (APSAC). The dose of APSAC was 0.1 U/kg, given intra-arterially (i.a.) into the carotid artery immediately proximal to the occlusive carotid artery thrombus. Lysis and restoration of carotid artery blood flow occurred in each of the 6 animals. However, reclosure occurred in all of the animals. Group B consisted of 6 animals that likewise were subjected to carotid artery injury and occlusive thrombus formation. As in the previous group, APSAC achieved successful thrombolysis in each of the animals. Within 5 minutes of having achieved thrombolysis, each animal received 7E3 monoclonal F(ab')2 antibody in an intravenous dose of 0.8 mg/kg. As indicated by light segments in each of the bars, carotid artery blood flow was maintained after inhibition of the platelet GPIb/IIa fibrinogen receptor. Group C included 5 animals. Occlusive carotid artery thrombosis was induced, and 30 minutes later, r-fibrolase in a dose of 4.0 mg/kg was infused intra-arterially immediately proximal to the occlusive arterial thrombus. Lysis of the occlusive lesion was achieved in each of the 5 animals. Five minutes after restoration of blood flow, each animal received 7E3 monoclonal F(ab')2 antibody in an intravenous dose of 0.8 mg/kg. Patency of the carotid artery was maintained in 4 of the 5 animals in the group (white portion of horizontal bars).
sus the right carotid arteries (infused with saline). r-Fibrolase resulted in a restoration of blood flow in the left carotid arteries (to about 70% of the preinjury state), whereas the right carotid arteries remained occluded after saline treatment. There was a significant 40% decrease in the weight of the residual thrombus in the left carotid artery of the animals treated with fibrolase plus 7E3 compared with the weight of thrombi retrieved from the right carotid arteries treated with 0.9% sodium chloride (23±7 versus 38±6 mg). Keeping in mind that the mean body weight of group C animals and thus the relative lumen size of the respective carotid arteries was less than that of the other two groups, invalidates a comparison among groups of respective carotid flow velocity and residual thrombus weights.

**Effects of r-Fibrolase on Physiological and Hematologic Parameters**

Tables 1 and 2 summarize the pertinent data with respect to the physiological and hematologic parameters measured in the experimental protocol. As indicated in Table 1, there are no changes in the circulating number of erythrocytes or platelets after treatment with r-fibrolase and 7E3. The hemoglobin and hematocrit values remain relatively constant during the observation period. There was an increase in the total WBC count in blood samples removed 120 minutes after the administration of fibrolase. The significance of this, if any, is not known at present.

The mean arterial blood pressure and the heart rate were not altered after the administration of r-fibrolase. Toward the end of the experimental protocol, however, mean arterial blood pressure declined very slightly below that of the initial control value. The relation of the decrease in mean arterial pressure to the administration of r-fibrolase is not readily apparent (Table 1).

The administration of r-fibrolase in a total infused dose of 4 mg/kg immediately proximal to the occlusive carotid artery thrombus did not alter the aPTT (Table 2) from its baseline value of 111±6 versus 113±18 seconds after r-fibrolase. Ex vivo platelet reactivity in response to arachidonic acid or ADP was decreased by approximately 60% in response to both agonists. Complete inhibition of ex vivo platelet reactivity was achieved after the administration of the 7E3 monoclonal F(ab')2 antibody (Table 2).

**Discussion**

Pharmacologic dissolution of an established thrombus has become an accepted therapeutic approach for many patients who develop thrombotic occlusive disease. Intravenous infusion of plasminogen activators including rt-PA, urokinase (UK), SK, and APSAC activate the fibrinolytic system and are effective in restoring blood flow in occluded arteries and veins.1,22

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**Table 1. Hematologic Measurements in the Anesthetized Dog Treated With Fibrolase and 7E3**

<table>
<thead>
<tr>
<th>Variable</th>
<th>RBCs, 10^6/mL</th>
<th>WBCs, 10^3/mL</th>
<th>PLTs, 10^9/mL</th>
<th>HGB, g/dL</th>
<th>HCT, %</th>
<th>Heart Rate, beats per minute</th>
<th>MAP, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>8±0.5</td>
<td>11±2</td>
<td>433±37</td>
<td>23±1</td>
<td>43±2</td>
<td>180±12</td>
<td>110±7</td>
</tr>
<tr>
<td>Fibrolase (5 minutes)</td>
<td>9±0.3</td>
<td>14±1</td>
<td>303±54</td>
<td>24±1</td>
<td>45±2</td>
<td>176±13</td>
<td>116±8</td>
</tr>
<tr>
<td>7E3 (30 minutes)</td>
<td>8±0.5</td>
<td>7±3</td>
<td>436±35</td>
<td>22±1</td>
<td>41±2</td>
<td>185±13</td>
<td>90±4</td>
</tr>
<tr>
<td>7E3 (120 minutes)</td>
<td>9±0.5</td>
<td>20±5</td>
<td>454±34</td>
<td>24±1</td>
<td>47±3</td>
<td>182±20</td>
<td>93±5</td>
</tr>
</tbody>
</table>

Data are mean±SEM, n=5.

RBCs indicates red blood cells; WBCs, white blood cells; PLTs, platelets; HGB, hemoglobin; HCT, hematocrit; MAP, mean arterial pressure.

Fibrolase was infused proximal to the mature thrombus in the left carotid artery, 4.0 mg/kg over 5 minutes. Five minutes after lysis, 7E3 was administered (0.8 mg/kg IV) to prevent carotid artery reocclusion.
SK, APSAC, and UK activate circulating plasminogen as well as fibrin-bound plasminogen within the thrombus. The widespread systemic activation of the fibrinolytic system leads to the depletion of α₂-antiplasmin (α₂-AP) and generation of free plasmin that degrades several plasma proteins including fibrinogen and factors V and VIII. Alternatively, rt-PA and recombinant single-chain UK-type plasminogen activator (scu-PA) activate plasminogen preferentially on the fibrin surface, where the fibrin-associated plasmin is protected from rapid inactivation by α₂-AP. Thus, fibrin specificity is considered to be of great importance in the development of effective and safe thrombolytic agents.22

Despite the development of more specific thrombolytic agents, currently available therapy has a number of important limitations. A significant percentage (25% to 30%) of patients with acute myocardial infarction are resistant to reperfusion within 90 minutes despite the use of potent thrombolytic agents or combinations.23 In addition, there is the potential for inducing systemic fibrinogenolysis with accompanying bleeding.24 Furthermore, a significant number of patients experience acute coronary reclosure after successful thrombolysis.25,26 The fibrin specificity of rt-PA and scu-PA in humans is not as pronounced as originally anticipated from animal models.27,28 Both rt-PA and scu-PA have relatively short half-lives due to rapid hepatic clearance, making it necessary to administer the agents by use of a loading dose followed by a continuous infusion, thereby consuming large amounts of material. Additionally, there is concern about the rapidly acting plasma inhibitor of t-PA, PAI-1,29 which is increased significantly in myocardial infarction30 and may, with other factors, predispose patients to reinfarction.31,32 Localization of PAI-1 to a thrombus by binding to fibrin could result in significant protection from degradation by plasminogen activator–type thrombolytic agents.17 Furthermore α₂-AP, which is cross-linked to fibrin by factor XIIIa, may account for a significant percentage of thrombi that are resistant to thrombolytic therapy,15 particularly in platelet-rich thrombi.16

Investigators have attempted to overcome the above-mentioned problems by enhancing thrombolytic activity and improving targeting to the thrombus. Thus, a new generation of thrombolytic agents has evolved including mutant forms of scu-PA and t-PA, hybrids between t-PA and scu-PA or between plasmin and t-PA, and synergistic combinations of t-PA and scu-PA.22,28 Despite these modifications, many of which must still be subjected to vigorous preclinical and clinical testing, all current thrombolytic agents depend on the generation of plasmin through activation of plasminogen. As such, they must be directed to a site where plasminogen exists; only after activating the endogenous source of plasminogen do they become effective.

In view of the potential limitations of plasminogen activator–based thrombolytic agents, alternative interventions having potent thrombolytic activity on their own or that may act synergistically with plasminogen activators need to be evaluated. In this report, we present investigations aimed at examining a mechanism of thrombolytic therapy different from presently available therapies, which are based on the activation of plasminogen. Snake venoms, particularly those from North American pit vipers, contain direct-acting fibrinolytic metalloproteinases.33 Fibrolase is the fibrinolytic enzyme from southern copperhead venom.12,13 Fibrolase represents one of the first highly effective thrombolytic agents that is not a plasminogen activator. The fibrinolytic action of fibrolase results from a direct proteolytic action on fibrin that is not susceptible to inhibition by serine proteinases known to interfere with plasminogen activator–based thrombolytic agents. Fibrolase is not dependent on any bloodborne intermediates for fibrin degradation because it acts by a completely different mechanism from plasminogen activators14 and acts rapidly and directly on thrombi with minimal alterations to the hemostatic system.34 Recent reports have demonstrated an association between the procoagulant response induced by plasminogen activator–based thrombolytic agents and the generation of plasmin.2–4 Formation of plasmin may, in concert with other factors, contribute to the 10% to 30% of patients who experience reclosure after "successful" coronary thrombolysis. In view of its mechanism of action, this should not be a potential side effect of r-fibrolase, which does not rely on plasminogen activation to degrade clot-associated fibrin.

Recent studies have revealed extensive sequence similarity between the venom fibrinolytic metalloproteinases and the closely related hemorrhagic metalloproteinases from similar snake venoms. The primary structure

<table>
<thead>
<tr>
<th>Time</th>
<th>Arachidonic Acid (0.325 mmol/L)</th>
<th>Arachidonic Acid (0.65 mmol/L)</th>
<th>ADP (5 μmol/L)</th>
<th>ADP (20 μmol/L)</th>
<th>aPTT, seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>79±3</td>
<td>84±3</td>
<td>78±3</td>
<td>84±4</td>
<td>24±1</td>
</tr>
<tr>
<td>Fibrolase, 4.0 mg/kg</td>
<td>5 minutes</td>
<td>34±14*</td>
<td>41±13*</td>
<td>14±4*</td>
<td>35±15*</td>
</tr>
<tr>
<td>7E3, 0.8 mg/kg</td>
<td>30 minutes</td>
<td>3±1*</td>
<td>4±1*</td>
<td>2±1*</td>
<td>3±1*</td>
</tr>
<tr>
<td></td>
<td>120 minutes</td>
<td>3±1*</td>
<td>4±2*</td>
<td>3±1*</td>
<td>4±2*</td>
</tr>
</tbody>
</table>

Data are mean±SEM, n=5.
Fibrolase was infused proximal to the mature thrombus, 4.0 mg/kg over 5 minutes. Five minutes after lysis, 7E3 was administered (0.8 mg/kg IV) to prevent carotid artery reocclusion.

*P<.05 vs respective baseline values.

Table 2. Ex Vivo Platelet Aggregations (%) to ADP and Arachidonic Acid in Before and After Fibrolase and 7E3 Administration in the Anesthetized Dog
of fibrolase exhibits approximately 60% sequence identity with several hemorrhagic and nonhemorrhagic snake venom metalloproteinases. However, fibrolase possesses no hemorrhagic activity, as demonstrated by lack of leakage of blood components into the surrounding tissue after injection of the enzyme under the skin of experimental animals. Although crude southern copperhead venom possessed significant hemorrhagic activity, purified fibrolase (at doses substantially higher than needed to detect this activity with purified hemorrhagic metalloproteinases) exhibited no detectable hemorrhagic activity. The lack of in vivo hemorrhagic activity is apparent with the recombinant enzyme as evidenced by the complete absence of internal bleeding observed upon gross examination of each of the dogs that received a total dose of 4 mg/kg.

To demonstrate in vivo thrombolytic efficacy of purified natural fibrolase, we investigated its activity in several animal thrombosis model systems. Previous studies revealed that infusion of fibrolase proximal to an occlusive thrombus produced rapid and effective thrombolysis in renal arterial and iliac venous thrombosis model systems. No bleeding or alterations in the hemostatic system were observed in treated animals. Further, no toxicity was observed and no angiographic, histologic, or functional evidence of injury was apparent. Additionally, the enzyme rapidly lysed 48-hour-aged clots in a venous thrombosis model, suggesting that one of the primary mechanisms of thrombus resistance to plasminogen activator–based thrombolytic therapy, aging of the thrombus, might not be applicable with the venom fibrinolytic enzyme. Also, since fibrolase does not require plasminogen, the enzyme would exhibit full activity when the circulating concentration of plasminogen is depressed. The early studies revealed that highly purified natural fibrolase, in the presence of low-dose heparin, produced rapid and consistent thrombolysis. More recently, the recombinant enzyme has become available, and we have examined its activity in a canine reoccluding carotid arterial thrombosis model system. Results described in the present communication are the first to fully document the in vivo thrombolytic potential of r-fibrolase. The present studies provide convincing evidence that r-fibrolase rapidly lyzes arterial thrombi. Although untoward side effects or toxicity were not apparent, one must withhold judgment on this until the metalloproteinase can be evaluated more completely under conditions where it is administered in a fully effective systemic dose. The primary intent of this investigation was to determine if r-fibrolase, a zinc-containing metalloproteinase, could achieve thrombolysis in an experimental animal model in which a platelet-rich, occlusive arterial thrombus develops in response to a deep arterial wall injury. The experimental model used is a modification of one originally developed in this laboratory and subsequently adapted to the carotid artery. The use of the carotid instead of the coronary artery has the advantage of greater yield of experimental data and where necessary allows the animal to serve as its own internal control, thereby facilitating the interpretation of results and simplifying statistical evaluation. This was an important consideration in the present study, in which r-fibrolase was in limited supply, thus accounting for the need to use relatively small animals and to administer the recombinant protein by local injection immediately proximal to the occlusive platelet-rich arterial thrombus. Furthermore, the limited supply of r-fibrolase precluded our desire to conduct dose-response studies to fully characterize the thrombolytic potential and pharmacokinetic properties of the metalloproteinase.

The study is not intended to compare r-fibrolase with other thrombolytic agents or to suggest that r-fibrolase possesses a therapeutic advantage over current therapeutic interventions used for thrombolysis. Because r-fibrolase shows enzymatic specificity for fibrin and acts independently of the need for plasmin generation, it was considered important to assess its in vivo capacity to induce arterial thrombolysis in an experimental model in which other known thrombolytic agents would achieve a comparable effect in lysing an occlusive arterial thrombus. For the latter purpose, we elected to use APSAC. As anticipated, APSAC resulted in successful thrombolysis and restoration of carotid artery blood flow in each of the animals to which it was administered. Without subsequent adjunctive therapy, however, rethrombosis followed within a relatively short time. The present results with APSAC on occlusive carotid artery thrombi and the need for adjunctive antplatelet therapy for the prevention of rethrombosis are identical to those of a previously published report from this laboratory.

The present preliminary studies indicate that r-fibrolase induced rapid thrombolysis but was not able to prevent rethrombosis in this reoccluding model system without adjunctive antplatelet therapy. Therefore, we combined the fibrinolytic enzyme with an antplatelet agent. We used the fibrinogen receptor antagonist 7E3, a monoclonal antibody to platelet GPIIb/IIIa (fibrinogen receptor). Because the GPIIb/IIIa receptor on the platelet surface represents the final common pathway for platelet aggregation, inhibition of this pathway represents an effective mechanism for inhibiting platelet aggregation independent of the inducing stimulus. That blood platelets serve as a promoter of rethrombosis in the experimental carotid artery thrombosis model was supported by studies that revealed that the platelet GPIIb/IIIa receptor antagonist SC-49992, a peptidomimetic of the fibrinogen sequence RGD (arginyl-glycyl-aspartyl-phenylalanine), which blocks binding to the platelet fibrinogen receptor, prevented rethrombosis, as did the snake venom–derived GPIIb/IIIa receptor antagonist apilaggin. A recent multicenter clinical trial (Thrombolysis and Angioplasty in Myocardial Infarction study, TAMI) demonstrated the efficacy of the 7E3 antibody in patients undergoing thrombolytic therapy for acute myocardial infarction.

Due to the limited availability of active material, the present study restricted the use of r-fibrolase to local administration immediately proximal to the site of the occlusive arterial thrombus. Although r-fibrolase enters the general circulation after dissolution of the left carotid arterial thrombus, there is minimal lysis of the contralateral thrombus (right carotid artery), presumably due to slow inactivation of the enzyme in the blood by α2-macroglobulin. Previous in vitro investigations have shown that fibrolase is inactivated by the α2-macroglobulin in a stoichiometric reaction. It is significant that APSAC, when studied in an identical fashion, involving local administration, like r-fibrolase also achieved lysis of the occlusive lesion. When used by local administration, neither r-fibrolase, as demon-
strated in this study, nor APSAC altered the aPTT from its baseline value.41 Willis and colleagues previously reported the purification45 and in vivo application46 of atroxase, a fibrinolytic enzyme from Crotalus atrox (western diamondback rattlesnake) venom. However, the latter studies used the intravenous route of administration for atroxase in a rat posterior vena cava thrombosis model system. The ability to monitor physiological functions and blood parameters was curtailed because of the small size of the animal species chosen for the studies.

The present study, although limited to the local application of r-fibrolase, demonstrates that local administration of the recombinant metalloproteinase lyses a carotid arterial thrombus rapidly and with no observable untoward systemic or hematologic sequelae. The enzyme in combination with antiplatelet therapy (7E3) offers a unique, safe, and specific mechanism for clot dissolution and may prove useful as a clinically efficacious alternative to, or for use in synergistic combination with, presently used thrombolytic agents.

Acknowledgments
This work was supported in part by the National Institutes of Health, Heart, Lung, and Blood Institute, grants HL-31389 (Dr Markland) and HL-19782-15 (Dr Lucchesi). The authors would like to acknowledge Drs Patricio Riquelme and Pablo Valenzuela, Chiron Corporation, Emeryville, Calif, for providing r-fibrolase.

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Circulation. 1994;90:2448-2456
doi: 10.1161/01.CIR.90.5.2448

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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