Synergistic Combinations of Recombinant Human Tissue-Type Plasminogen Activator and Human Single-Chain Urokinase-Type Plasminogen Activator

Effect on Thrombolysis and Reocclusion in a Canine Coronary Artery Thrombosis Model With High-Grade Stenosis

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The synergistic effects of recombinant human tissue-type plasminogen activator (rt-PA) and single-chain urokinase-type plasminogen activator (scu-PA) on coronary arterial thrombolysis were investigated in open-chest dogs with thrombosis of the left anterior descending coronary artery and a superimposed high-grade stenosis. A 90% stenosis was generated by external constriction, reducing blood flow to 40±10% of baseline. Localized thrombosis was produced by endothelial cell injury and instillation of thrombin and fresh blood. Intravenous infusion for 60 minutes of either 30 μg/kg/min rt-PA alone or 10 μg/kg/min scu-PA alone consistently produced coronary artery recanalization (six of eight dogs and five of five dogs, respectively) but was almost always associated with reocclusion during or shortly after the end of the infusion (four of six dogs and five of five dogs, respectively). Infusion of either 15 μg/kg/min rt-PA or 5 μg/kg/min scu-PA for 60 minutes did not cause coronary artery recanalization (none of four dogs in each group). Combined infusion of 7.5 μg/kg/min rt-PA and 2.5 μg/kg/min scu-PA for 60 minutes (one fourth of the minimum thrombolytic dose of each agent) induced coronary artery recanalization (six of six dogs) but was also associated with early reocclusion (six of six dogs). Combined infusion of 3.75 μg/kg/min rt-PA and 1.25 μg/kg/min scu-PA for 60 minutes did not consistently cause recanalization (one of four dogs). Combined infusion of 15 μg/kg/min rt-PA and 5 μg/kg/min scu-PA for 60 minutes caused recanalization in all of six dogs but was associated with reocclusion in all six. Pathologic examination revealed that reocclusion was caused by platelet-rich thrombotic material. Bolus injection of 0.6 mg/kg of a monoclonal antibody (7E3) directed against the platelet GPIIb/IIIa receptor 10 minutes before the start of a combined infusion of 7.5 μg/kg/min rt-PA and 2.5 μg/kg/min scu-PA for 60 minutes abolished reocclusion. It is concluded that rt-PA and scu-PA used in equipotent fractional combinations act synergistically on coronary arterial thrombolysis in this dog model. As observed previously in a rabbit venous thrombosis model, synergism only occurs in a relatively narrow concentration range representing twofold to fourfold pharmacologic synergism. Furthermore, in the presence of high-grade stenosis, recanalization with the synergistic combination is consistently associated with reocclusion that can be prevented with the use of a potent monoclonal antiplatelet GPIIb/IIIa antibody. The combination therapy thus may allow a reduction of the total thrombolytic dose but does not eliminate the problem of reocclusion. (Circulation 1989;79:393–399)
Human tissue-type plasminogen activator (t-PA) and single-chain urokinase-type plasminogen activator (scu-PA) activate the blood fibrinolytic system with a significant but not absolute degree of clot specificity.1 However, to obtain rapid coronary artery recanalization in patients with acute myocardial infarction, large doses of either thrombolytic agent (50–100 mg) must be rapidly infused. Such high infusion rates result in a 1,000-fold increase of the plasma levels of t-PA and scu-PA, whereby the fibrin-specificity of thrombolysis is lost in some patients.2,3

We have previously reported that t-PA and scu-PA act synergistically on thrombolysis in rabbits with jugular vein thrombosis.4 This synergism was confirmed in a small number of patients with myocardial infarction and angiographically confirmed coronary artery thrombosis.5,6 The existence of in vivo synergism between t-PA and scu-PA might permit a significant reduction of the total amount of material needed for thrombolysis, diminishing the side effects associated with currently used high-dose infusion regimens.

The optimal application of synergism of t-PA and scu-PA for coronary arterial thrombolysis and its impact on maintaining stable coronary patency remain to be established. One major difficulty in the continued investigation of this phenomenon is that synergism of rt-PA and scu-PA in approximately equifractional combinations cannot adequately be reproduced in in vitro systems.6

In the present study, we have evaluated the potency of combined infusions in varying doses of recombinant t-PA and natural scu-PA obtained from a kidney cell culture with respect to both coronary arterial thrombolysis and early reocclusion in a canine preparation that simulates coronary artery occlusion in patients with acute myocardial infarction. Studies on coronary artery reocclusion after the use of this combination were also performed.

**Methods**

**Thrombolytic and Antiplatelet Agents**

t-PA was obtained from Genentech, South San Francisco, California, as a lyophilized powder and consisted predominantly of the single-chain form (G11044) with a specific activity of approximately 600,000 IU/mg as compared with the International Reference Preparation for t-PA, scu-PA, obtained from a transformed human kidney cell line, was provided by Sandoz, Basel, Switzerland, in a liquid formulation containing 0.75 mg/ml, with a specific activity of approximately 80,000 IU/mg as compared with the International Reference Preparation for urokinase, and was kept frozen until use. F(ab')2 fragments of the murine monoclonal antibody (7E3), directed against the platelet receptor GPIIb/IIIa were supplied by Centocor, Malvern, Pennsylvania.

**Experimental Preparation**

Coronary artery thrombosis and endothelial cell damage were produced in dogs as previously described.8 In addition, superimposed high-grade stenosis was produced by the application of an external constrictor, and blood flow was continuously monitored with an electromagnetic flow probe.7

Adult mongrel dogs weighing 15–20 kg were anesthetized with pentobarbital (30 mg/kg i.v.) and received additional doses as required. The dogs were intubated and placed on a respirator. Procainamide (1.5 g i.m.) and lidocaine (2–3 mg/min i.v.) were given as prophylactic antiarrhythmic therapy. The left carotid artery was exposed through an incision in the neck. A modified No. 7-1 Amplatz coronary angiography catheter was placed in the ascending aorta. A thoracotomy was performed via the left fifth intercostal space, and the left internal mammary artery was cannulated for continuous blood pressure monitoring. The pericardium was incised and suspended to create a pericardial cradle. Epicardial electrocardiographic leads were placed, and the electrocardiogram was continuously monitored. A 1.5-cm long segment of the left anterior descending coronary artery (LAD) was isolated, distal to the septal artery, and any large proximal diagonal branches and side branches were ligated. A catheter (0.7 mm i.d.) was inserted into a side branch of the isolated LAD segment. An electromagnetic flow probe (FM501, Carolina Medical Electronics, King, North Carolina) was placed on the proximal LAD for continuous monitoring of blood flow.

A selective angiogram of the LAD was obtained with the use of 1–2 ml meglumine diatrizoate and recorded on videotape. One milliliter of blood was drawn for later use in forming the thrombus. The dog was heparinized with 150 units/kg i.v. heparin, followed by 50 units/kg/hr to maintain the activated partial thromboplastin time from two to two and one half times that baseline. The procedure for producing the constriction to reduce blood flow to 40% of baseline was as follows. The electromagnetic flow probe was placed around the proximal LAD coronary artery, and the baseline flow was measured. The constrictor, which consisted of a 2-mm wide plastic belt with grooves, permitting progressive reduction of the circumference, was placed around the LAD near the distal end of the segment and stepwise constricted until the flow probe revealed a flow reduction to approximately...
40%. Then an angiogram was performed to confirm the presence of antegrade flow with clearing of contrast medium within four heart beats.

Snare occlusions 1 cm apart were made distal to the flow probe and proximal to the constriction site, and the segment was emptied of blood via the cannulated side branch. The isolated LAD segment was traumatized by external compression with blunt forceps (four times for 3–5 seconds) to produce endothelial cell injury and to promote thrombus adherence to the luminal surface. The segment was flushed by injecting saline through the cannulated side branch, and the segment was again isolated and emptied. Thrombin (0.1 ml 100 units/ml solution) and the stored blood (0.3 ml) were injected through the side branch catheter into the empty coronary artery segment. After 5 minutes, the proximal snare was released; 2 minutes later, the distal tourniquet was released. An angiogram was performed 30 minutes after thrombus formation to confirm total occlusion in association with the complete absence of antegrade flow as demonstrated by the electromagnetic flow probe.

Infusion of the thrombolytic agents was carried out for 60 minutes via a hind leg vein with an infusion pump (Harvard Apparatus, South Natick, Massachusetts). When rt-PA and scu-PA were infused simultaneously, they were delivered into separate venous sites. The electromagnetic flow probe was used for continuous monitoring of antegrade flow in the LAD. Angiograms were performed every 15 minutes to monitor occlusion and in addition when the flow probe showed evidence of reperfusion or reocclusion. Monitoring of blood flow continued for 30 minutes after completion of the infusion.

At completion of the study, the animal was killed with an overdose of pentobarbital, and the heart was fixed with 5% formaldehyde for pathologic examination. The thrombosed, the stenotic, and the poststenotic LAD segments were removed intact, sectioned at 2-mm intervals, and routinely processed for histology. Sections were stained with hematoxylin and eosin and examined by light microscopy for the presence of luminal or mural thrombus as described earlier.7

### Drug Infusion Regimens

rt-PA was infused at rates of 15 or 30 μg/kg/min and scu-PA at doses of 5 or 10 μg/kg/min to reestablish the minimum thrombolytic dose. These infusion rates were deduced from our earlier experience with these agents in dogs with coronary artery occlusion.8,9 The synergistic effects of rt-PA and scu-PA on coronary arterial thrombolysis were investigated by infusing the following combinations: 3.75 μg/kg/min rt-PA and 1.25 μg/kg/min scu-PA (one eighth of the minimum thrombolytic doses), 7.5 μg/kg/min rt-PA and 2.5 μg/kg/min scu-PA (one fourth of the minimum thrombolytic doses), and 15 μg/kg/min rt-PA and 5 μg/kg/min scu-PA (one half of the minimum thrombolytic doses). An additional group of dogs, infused with 7.5 μg/kg/min rt-PA and 2.5 μg/kg/min scu-PA for 60 minutes, was injected with a bolus of 0.6 mg/kg F(ab')2 fragments of the antiplatelet GPIIb/IIIa antibody 7E3 10 minutes before the start of the combined infusion of the thrombolytic agents. A total of 43 dogs were studied.

### Blood Analyses

Venous blood samples for determination of the levels of fibrinogen, rt-PA– and scu-PA–related antigen, and activated partial thromboplastin time were drawn into 0.01 M citrate containing 150 kallikrein inhibitor units/ml aprotinin before the infusion; 25 and 50 minutes after the start of the infusion; and at 5, 10, 20, and 30 minutes after end of the infusion. The blood samples were kept on ice until the end of the experiment, and then centrifuged at room temperature for 10 minutes; the plasma was stored at −20°C until analyzed. The assays were performed as previously described.8-10 The coagulation rate assay,11 performed as described by Vermylen et al.,12 is insensitive to heparin concentrations up to 10 units/ml plasma.13 Serial foreleg bleeding times were performed on those dogs receiving the antiplatelet antibody 7E3.

### Results

#### Coronary Thrombolysis

The results of coronary thrombolysis are summarized in Table 1. The external constriction of the LAD caused a reproducible reduction in coronary

### Table 1. Coronary Artery Thrombolysis With rt-PA, scu-PA, and Combinations

<table>
<thead>
<tr>
<th>Dose of thrombolytic agent</th>
<th>Poststenotic blood flow</th>
<th>Reperfusion</th>
<th>Reocclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt-PA (μg/kg/min)</td>
<td>scu-PA</td>
<td>n</td>
<td>Time (min)</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>3.75</td>
<td>1.25</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>7.5</td>
<td>2.5</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are mean ±SD.
artery blood flow to approximately 40% of the baseline value. This corresponds to a stenosis of 90% as evidenced by postmortem arteriography (not shown).

Infusion of rt-PA at a rate of 15 µg/kg/min for 60 minutes in four dogs did not induce recanalization within 1 hour. With 30 µg/kg/min for 60 minutes in eight dogs, recanalization was obtained in six dogs after 28 ± 30 minutes (mean ± SD), but reocclusion occurred in four of these six animals within 6 ± 5 minutes. Infusion of scu-PA at a rate of 5 µg/kg/min was ineffective in all of four dogs, whereas 10 µg/kg/min caused recanalization in five of six dogs within 38 ± 14 minutes. However, cyclic reocclusion occurred in all dogs, within 7 ± 5 minutes. Thus, infusion rates of 30 µg/kg/min rt-PA and 10 µg/kg/min scu-PA appear to be the minimum dosages required to consistently attain coronary artery recanalization within 60 minutes. The phenomenon of cyclic recanalization and reocclusion is represented in Figure 1.

The combination of 3.75 µg/kg/min rt-PA and 1.25 µg/kg/min scu-PA (one eighth of the minimum thrombolytic doses) infused simultaneously for 60 minutes produced recanalization in only one of four dogs, and this was followed by rapid reocclusion. Combined infusion of 7.5 µg/kg/min rt-PA and 2.5 µg/kg/min scu-PA (one fourth of the minimum thrombolytic dosages) caused coronary arterial recanalization in all of six dogs studied, within 36 ± 16 minutes, but in each case reocclusion occurred during or shortly after the end of the infusion. With the combination of 15 µg/kg/min rt-PA and 5 µg/kg/min scu-PA (one half of the minimum thrombolytic dosages), coronary arterial recanalization occurred in all of six animals, but reocclusion cycles were still observed in all but one of the animals (Figure 1).

In five dogs, 0.6 mg/kg of the F(ab')2 fragment of the murine monoclonal antibody 7E3 was injected 10 minutes before the start of a combined infusion of 7.5 µg/kg/min rt-PA and 2.5 µg/kg/min scu-PA. This resulted in recanalization in all dogs, within 25 ± 13 minutes, and was associated with virtually complete elimination of the cyclical reocclusion phenomenon (Figure 1).

**Blood Analyses**

Infusion of rt-PA, scu-PA, or their combination did not cause extensive systemic activation of the fibrinolytic system as evidenced by the negligible-to-minor changes in the lowest fibrinogen levels.
TABLE 2. Residual Fibrinogen and Antigen Levels During Infusion of rt-PA and scu-PA

<table>
<thead>
<tr>
<th>Dosage of thrombolytic agent (μg/kg/min)</th>
<th>Residual fibrinogen (% of baseline)</th>
<th>rt-PA antigen (μg/ml)</th>
<th>scu-PA antigen (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt-PA scu-PA n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>82±8</td>
<td>1.7±0.46</td>
<td>NA</td>
</tr>
<tr>
<td>30</td>
<td>83±6</td>
<td>1.6±0.60</td>
<td>NA</td>
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<tr>
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<td>91±16</td>
<td>NA</td>
<td>0.91±0.25</td>
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<tr>
<td>...</td>
<td>87±12</td>
<td>NA</td>
<td>1.9±0.53</td>
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<tr>
<td>3.75</td>
<td>93±13</td>
<td>0.40±0.13</td>
<td>ND</td>
</tr>
<tr>
<td>7.5</td>
<td>92±5</td>
<td>0.97±0.76</td>
<td>0.29±0.15</td>
</tr>
<tr>
<td>15</td>
<td>83±4</td>
<td>1.9±0.88</td>
<td>0.25±0.17</td>
</tr>
</tbody>
</table>

Values are mean ± SD.
ND, not determined; NA, not applicable.
The rt-PA and scu-PA antigen levels were determined before the end of the infusion. The fibrinogen level was determined 30 minutes after the end of the infusion.

(Table 2). During infusion, plateau levels of rt-PA- and scu-PA–related antigen in plasma were reached proportional to the infusion rates (Table 2).

In the dogs injected with 0.6 mg/kg 7E3-F(ab')_, in addition to the combined infusion of 7.5 μg/kg/min rt-PA and 2.5 μg/kg/min scu-PA, the fibrinogen and rt-PA antigen levels were similar to those in the group without the antibody (78±22% and 0.91±0.43 μg/ml, respectively). However, the bleeding time in the group with antibody was markedly prolonged at the end of the infusion (from 4.8±1.2 to 18±12 minutes).

Pathologic Analyses

Microscopic examination of the LAD segments of dogs with coronary artery recanalization followed by acute reocclusion revealed occlusive thrombus rich in fibrin and platelets (Figure 2A). In dogs with persistent recanalization, only small amounts of residual mural thrombus and platelets (Figures 2B and 2C) were observed in association with the extensive intimal damage.

Discussion

This study was undertaken to investigate the thrombolytic potency of varying doses of rt-PA and scu-PA, infused separately or in combination, with a canine model of coronary thrombosis superimposed on high-grade coronary artery stenosis. This model was selected because it simulates important features of coronary artery thrombosis in patients with acute myocardial infarction, namely, endothelial cell damage with thrombosis and superimposed high-grade coronary artery stenosis. We have previously shown that this model also simulates the phenomenon of coronary artery reocclusion following successful thrombolysis, despite full anticoagulation with heparin, as a result of deposition of platelet- and fibrin-rich thrombotic material at the site of the luminal constriction.14

Our present findings confirm that rt-PA and scu-PA act synergistically in vivo as determined by strict pharmacologic analysis.6,15 Synergism between rt-PA and scu-PA, which was previously demonstrated in a rabbit jugular vein thrombosis model, is now extended to a coronary artery thrombosis model with superimposed high-grade stenosis in the dog, indicating that it is not a species-specific phenomenon, as was recently suggested for rt-PA and urokinase.16 The concentration range in which synergism is observed in rabbits and dogs is comparable. While the combination of 15% rt-PA and 15% scu-PA was equivalent with that of the individual agents in the rabbit model, we observed clear synergism with a combination of 25% of the minimum thrombolytic dose of each but not with 12.5% of each in the coronary artery thrombosis model in the dog. Preliminary studies in patients with coronary artery occlusion have indicated that the extent of synergism may be somewhat more pronounced in humans6 where it can be demonstrated with equipotent algebraic fractions of 0.1–0.2.

The synergistic combinations of rt-PA and scu-PA did not induce significant fibrinogen breakdown in the dog, confirming that the combined infusions have no synergistic effect on systemic activation of the fibrinolytic system. Extrapolation of this observation to patients can only be made with some caution, because of the higher intrinsic sensitivity of the human plasma fibrinolytic system to systemic activation by rt-PA or scu-PA.

Neither rt-PA nor scu-PA alone, nor their synergistic combination, prevented coronary artery reocclusion by platelet-rich thrombus. However, reocclusion was effectively eliminated with a monoclonal antibody that profoundly inhibits platelet aggregation.17 This indicates that coronary artery reocclusion in the presence of high-grade stenosis is primarily dependent on platelet deposition and explains why reocclusion is not adequately prevented by either heparin anticoagulation14,17 or with the use of nonselective thrombolytic agents that cause severe systemic fibrinogen depletion.18 The apparent inability of single or combined infusions of thrombolytic agents to prevent coronary artery reocclusion, therefore, does not reside in their inability to lyse fibrin but in their inefficient interference with the process of platelet aggregation. Therefore, the
Figure 2. Cross-sections of the prestenotic segments of the left anterior descending coronary artery. Panel A: From a dog given 7.5 μg/kg/min rt-PA and 2.5 μg/kg/min scu-PA, resulting in reperfusion followed by reocclusion. Extensive intimal and medial damage and intraluminal thrombus (T) rich in fibrin and platelets are revealed. (Magnification, ×16; stain, hematoxylin and eosin.) Panel B: From a dog given 15 μg/kg/min rt-PA and 5 μg/kg/min scu-PA, resulting in stable reperfusion. Extensive intimal and medial damage but minimal thrombus are present. (Magnification, ×16; stain, hematoxylin and eosin.) Panel C: Higher magnification of same arterial cross-section shown in Panel B. Note the focally detached internal elastic lamina (arrows), the absence of endothelial cells, and the presence of red blood cells and fibrin on the intimal surface. Medical injury is also apparent by the lack of smooth muscle nuclei. (Magnification, ×125; stain, hematoxylin and eosin.)
use of a maintenance infusion of thrombolytic agents for the prevention of reocclusion, although highly effective in humans when given at a sufficiently high infusion rate,8,19 may not represent the optimal biologic solution to this problem and, in addition, may increase the bleeding risk. An alternative and more rational approach would involve the use of a combined minimal effective dose of synergistic thrombolytic agents to efficiently remove fibrin without causing systemic fibrinogen breakdown in association with antiplatelet blockade by potent and specific agents to prevent coronary artery reocclusion. Additional, more-detailed investigation of the interaction between synergistic combinations of thrombolytic agents and antiplatelet agents, in the setting of coronary artery thrombosis with high-grade stenosis, seems to be warranted to obtain maximal stable coronary artery recanalization with minimal associated toxicity.

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References

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