Acceleration of Recombinant Tissue-Type Plasminogen Activator–Induced Thrombolysis and Prevention of Reocclusion by the Combination of Heparin and the Arg-Gly-Asp–Containing Peptide Bitistatin in a Canine Model of Coronary Thrombosis

Ronald J. Shebuski, Inez J. Stabilito, Gary R. Sitko, and Mark H. Polokoff

The effect of tissue-type plasminogen activator (t-PA) alone or in combination with heparin, the Arg-Gly-Asp–containing peptide bitistatin, or both heparin and bitistatin was evaluated on thrombolysis time and acute reocclusion in a canine model of coronary thrombosis. Thrombus formation was elicited by electrolytic injury with a needle electrode to the endothelial surface of the circumflex coronary artery in the open-chest, anesthetized dog in the presence of a flow-limiting critical stenosis. Thirty minutes after spontaneous coronary artery occlusion, t-PA (1 mg/kg i.v. over 90 minutes) was administered. Group 1 was given t-PA alone; reperfusion occurred at 78.2±5.6 minutes with a reperfusion incidence of 60% (6/10). Group 2 received t-PA plus heparin (100 units/kg plus 50 units/kg/hr); reperfusion occurred at 61.9±9.1 minutes with a reperfusion incidence of 90% (9/10). Group 3 received t-PA plus heparin plus bitistatin (30 μg/kg plus 3 μg/kg/min); reperfusion occurred at 47.3±7.6 minutes (p<0.05 versus group 1) with a reperfusion incidence of 90% (9/10). Group 4 received t-PA plus bitistatin, and reperfusion occurred at 51.8±8.5 minutes; however, the reperfusion incidence was only 60% (6/10). In groups 1, 2, and 4, acute reocclusion occurred in more than 80% of the reperfused dogs, whereas in group 3 reocclusion occurred in 22% (2/9) of the reperfused dogs (p<0.05 versus group 1). The dose of heparin used in this study increased activated partial thromboplastin times 1.5–2.0-fold over control. Bitistatin elicited a fourfold increase in bleeding time with almost complete suppression of platelet aggregation to ADP, collagen, and U46619. Residual thrombus wet weights, which were determined at the end of each experiment, were significantly lower only for group 3 (2.0±0.4 mg) compared with control group 1 (5.0±0.8 mg). These data demonstrate, in this model of coronary thrombosis in the canine, that the Arg-Gly-Asp–containing peptide bitistatin accelerates t-PA–induced thrombolysis and in combination with heparin prevents acute thrombotic reocclusion. (Circulation 1990;82:169–177)

Reocclusion of coronary arteries after successful thrombolytic therapy is a persistent clinical problem with a reported incidence of 20–30%.1,2 The patients at the greatest risk of acute reocclusion are those with a high-grade residual stenosis after thrombolytic therapy of 80% or greater.3 Thus, prevention of reocclusion with adjunctive pharmacologic agents is an area that is being actively studied with numerous approaches involving anticoagulants, antiplatelet agents, and maintenance infusions of thrombolytics.3–5

The factor(s) responsible for acute reocclusion has not been precisely identified. Thus, because thrombus is primarily comprised of fibrin-bound erythrocytes and platelets, it has seemed prudent to administer heparin alone or in combination with antiplatelet agents such as aspirin. However, even in the presence of full heparin anticoagulation and aspirin treatment, reocclusion still occurs in a high percentage of patients.1 Therefore, strategies other than aspirin are being evaluated, primarily in animal models, in order to design a completely effective therapy.
models of experimental thrombosis and thrombolysis, for reduction in the incidence of acute reocclusion.

Thromboxane-receptor antagonists, as well as thromboxane synthase inhibitors, have been demonstrated to prevent postlysis reocclusion in animal models of coronary thrombosis. This action may be related to platelet activation and subsequent release of platelet-derived thromboxane A2 in response to tissue-type plasminogen activator (t-PA) or streptokinase infusion, as demonstrated in humans and animals. However, these approaches do not take into account platelet activation in response to agents other than thromboxane A2, such as collagen, serotonin, and thrombin. Thus, in an attempt to inhibit platelet aggregation independent of the nature of the mediator, agents directed against the platelet surface receptor for fibrinogen, such as glycoprotein IIb/IIIa, have been developed. Platelet binding of fibrinogen, by means of the IIb/IIIa-Receptor complex, is a final common pathway of platelet aggregation leading to thrombus formation.

In an approach to inhibit platelet aggregation independent of the nature of the platelet agonist, Coller et al. developed a murine monoclonal antibody (7E3) that was directed against the platelet glycoprotein IIb/IIIa complex. This antibody was demonstrated to produce an antithrombotic effect in dogs and monkeys when infused intravenously. However, 7E3 antibody was then assessed in combination with t-PA in dogs and was shown to accelerate arterial thrombolysis and prevent thrombotic reocclusion. However, 7E3 did not potentiate t-PA in a canine model of venous thrombosis presumably due to the lack of extensive platelet involvement in this experimental preparation. A different, but related, antibody (LJ-CP8) was injected into baboons with Dacron vascular grafts; positive effects were exhibited in one study, and negative effects were exhibited in another.

Other approaches to inhibit fibrinogen binding to glycoprotein IIb/IIIa involve the use of small synthetic peptides that contain the tripeptide Arg-Gly-Asp sequence (RGD) or natural RGD-containing proteins derived from snake venoms, such as trigramin, echistatin, or bitistatin. RGD-containing viper venom peptides are several hundred times more potent antiplatelet agents as compared with RGD and show similar binding affinity to platelet fibrinogen receptors as monoclonal anti-glycoprotein IIb/IIIa antibodies. Bitistatin is an 83-amino acid protein that contains an RGD sequence at residues 64–66. Bitistatin was derived from the venom of the viper Bitis arietans and purified to greater than 90% homogeneity. We have previously characterized bitistatin pharmacologically in a canine model of repetitive thrombus formation in stenosed coronary arteries, such that we could establish an effective dose to use in combination with t-PA. Thus, the aim of this study was to determine the ability of bitistatin alone and in combination with heparin to influence the rate of t-PA–induced thrombolysis as well as to prevent thrombotic reocclusion in a canine model of coronary artery thrombosis.

Methods

Thrombosis/Thrombolysis

Mongrel dogs of either sex (9–18 kg) were anesthetized with pentobarbital sodium (30 mg/kg i.v.), intubated, and ventilated with room air at a tidal volume of 20 ml/kg and a frequency of 12 inflations/min (Harvard respirator, Harvard Apparatus, South Natick, Mass.). The right femoral artery and vein were exposed, and catheters were inserted for monitoring arterial blood pressure (Statham P23 1D pressure transducer, Gould Inc., Glen Burnie, Md.) and administration of drugs, respectively. The left external jugular vein and the left saphenous vein were also exposed, and catheters were inserted for continuous infusion of 5% dextrose in saline and infusion of drugs, respectively. A left thoracotomy was performed at the fifth intercostal space; the heart was exposed and suspended in a pericardial cradle. A 2-cm section of the left circumflex coronary artery was isolated proximal to the first obtuse marginal branch and instrumented proximal to distal with an electromagnetic flow probe (model 501, Carolina Medical Electronics, Inc., King, N.C.), a stimulation electrode, an adjustable mechanical occluder (Goldblatt’s clamp), and a silk snare (Figure 1). The stimulation electrode was constructed from a 26-gauge stainless steel hypodermic needle tip attached to a 30-gauge Teflon-insulated silver-coated copper wire. The mechanical occluder was constructed of stainless steel with a stainless steel screw (2-mm diameter), which could be manipulated to control...
vessel circumference. Before the administration of any experimental drugs, the occluder was sufficiently tightened around the artery to eliminate the reactive hyperemic response without affecting resting left circumflex coronary blood flow.

Continuous recordings of systemic blood pressure and mean and phasic left circumflex coronary artery blood flow were displayed on a model 7E polygraph (Grass Instrument Co., Quincy, Mass.). Zero flow and hyperemic flows were determined by occluding the circumflex coronary artery distal to the flow probe for 20 seconds with the snare.

Thirty minutes after surgical preparation, a 150-μA continuous anodal direct current stimulation was initiated and maintained until left circumflex coronary artery blood flow decreased to zero flow and remained there. Direct electrical stimulation was delivered by a Grass constant-current unit (model CCUIA), a Grass stimulus-isolation unit (model SIU5B), and a Grass stimulator (model S48) connected to the intraluminal coronary artery electrode. This model consistently resulted in spontaneous occlusion due to thrombus formation that was responsive to thrombolytic agents. t-PA elicited reperfusion followed by spontaneous thrombotic reclosure in a high percentage of dogs (Figure 2).

Ex Vivo Platelet Aggregation

Blood was withdrawn from the femoral artery of the dog into a plastic syringe containing one part 3.8% trisodium citrate to nine parts blood. Platelet-rich plasma was obtained by centrifugation of this mixture at 150g for 10 minutes at room temperature. Platelet count was adjusted to 300,000/mm³. All aggregation studies were performed in an aggregometer (Chrono-log Corp., Havertown, Pa.) by using 0.25 ml platelet-rich plasma in a siliconized cuvette stirred at 1,000 rpm. Platelet-rich plasma was prewarmed for 2 minutes at 37°C before addition of agonist. ADP, collagen, and U46619 of different molar concentrations were added to the cuvette, and the change in light transmission was measured to the point where the tracing reached a plateau. Platelet aggregation is expressed as the percentage of light transmittance, with platelet-poor plasma representing 100% light transmittance.

Determination of the Effect of t-PA on Plasma Fibrinogen

Blood for the measurement of fibrinogen concentration was anticoagulated with 3.8% sodium citrate (9 vol blood to 1 vol anticoagulant) and centrifuged at 4°C at 1,200g for 15 minutes; the platelet-free plasma was stored at −70°C until it was assayed.

The fibrinogen concentration was determined by using the Electra 800 automated clot timer (Medical Laboratory Automation, Mt. Vernon, N.Y.) and commercially available reagents (American Dade, Aquada, Puerto Rico). The fibrinogen assay was based on the von Clauss thrombin-clotting method.31

Measurement of Bleeding Times

Bleeding time was measured with the Simplate bleeding time device (Onganon Teknika Corp., Durham, N.C.). Uniform incisions were made with the Simplate on the mucous membrane of the inner upper lip of the dog. The bleeding of the incision was timed from the moment the puncture was made to the moment when the bleeding stopped.

Materials

t-PA (Activase) was purchased from Genentech, South San Francisco, Calif., and was diluted to a final concentration of 2 mg/ml with sterile water supplied by the manufacturer. Bitistatin was derived from the venom of the viper Bitis arietans and purified to greater than 90% homogeneity.25 The other 10% of the bitistatin preparation was N-2 bitistatin, representing an 81- amino acid species. Bitistatin was lyophilized and reconstituted daily in sterile saline at a concentration of 1 mg/ml. ADP and collagen were purchased from Chrono-log, and U46619 and sodium heparin were purchased from The Upjohn Co., Kalamazoo, Mich.

Statistical Analysis

Results are expressed as mean±SEM. Hemodynamic parameters were analyzed by one-way analysis of variance, followed by a Dunnett’s test when the overall F ratio was significant. Homogeneity of variances was verified using Levene’s test, and a weighted analysis of variance was performed when unequal variances were detected. Data collected for multiple samples were analyzed by repeated-measures analysis of variance. Missing observations were estimated by least-squares techniques, and sample sizes were compared with baseline by using Dunnett’s procedure.
Results

Electrolytic thrombus formation was induced in 45 dogs in this study. Four groups of drug-treated animals of 10 each were randomly studied (groups 1–4) as described in Figure 3; an additional five dogs received no t-PA or adjunctive treatment (group 5). Group 1 consisted of dogs receiving t-PA alone (100 μg/kg plus 10 μg/kg/min i.v. for 90 minutes) administered 30 minutes after spontaneous electrically induced thrombus formation. After termination of t-PA, the dogs were followed for an additional 2 hours to determine the incidence of reocclusion. Group 2 dogs were given heparin (100 units/kg i.v.) 15 minutes after the occlusion, followed 15 minutes later by t-PA. Heparin was also administered every hour after the initial bolus at a dose of 50 units/kg i.v. Group 3 dogs received heparin and bitistatin (30 μg/kg plus 3 μg/kg/min i.v.) 15 minutes after the thrombotic occlusion, followed 15 minutes later by t-PA. Group 4 dogs received bitistatin 15 minutes after the occlusion, followed 15 minutes later by t-PA. At the end of each experiment the coronary artery was removed from the dog, and the thrombus wet weight was determined. Throughout the course of the experiment, blood samples were obtained as described in the protocol (Figure 3) for analysis of platelet aggregation, activated partial thromboplastin time (groups 2 and 3 only), and fibrinogen levels. ADP-induced platelet aggregation was assessed in only the first five animals of groups 1–4. At the same time points, bleeding times were also determined. Dogs from all groups had similar body weights with a range of 9–18 kg.

Dogs in groups 1–5 had similar resting coronary blood flows and, before placement of the Goldblatt clamp on the coronary artery, had a normal reactive hyperemic response after a brief, 20-second mechanical occlusion (data not shown). On placement of the Goldblatt clamp onto the coronary artery, resting blood flow was affected minimally; however, the reactive hyperemic capability of the circumflex coronary bed was essentially eliminated. We have previously observed that this level of obstruction (almost complete elimination of the reactive hyperemia) is necessary to elicit consistent reocclusion after successful t-PA–induced thrombolysis in this model.7

On electrical stimulation of the circumflex coronary artery, spontaneous occlusion occurred in groups 1–4 with a mean range of times varying from 45 to 84 minutes (Table 1). The incidence of t-PA–induced reperfusion in group 1 dogs was only 60%, whereas treatment with heparin (group 2) or heparin and bitistatin (group 3) increased the incidence of reperfusion to 90% (Table 1). Bitistatin alone (group 4) did not increase the incidence of reperfusion; thus, this effect appears to be related exclusively to the addition of heparin to the experimental protocol. None of the dogs in group 5 (n = 5) underwent spontaneous lysis in the absence of t-PA infusion.

![Diagram of protocol used in this investigation. Fifteen minutes after spontaneous occlusion (Occ), either heparin (Hep), heparin and bitistatin (Hep/Bitis), or bitistatin (Bitis) is administered at indicated doses; 15 minutes later, tissue-type plasminogen activator (t-PA) (1 mg/kg, total dose) is administered. Blood samples for platelet aggregation, activated partial thromboplastin time, and fibrinogen levels are taken throughout the experimental protocol (samples I–IV). At termination of the experiment, thrombus is excised and weighed.](http://circ.ahajournals.org/lookup/fig/1/6/8/1/2/172/I.png)

**Figure 3.**

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**Table 1. Characteristics of Coronary Blood Flow in Response to Thrombosis, Thrombolysis, and Rethrombosis in Canine Model of Coronary Thrombosis**

<table>
<thead>
<tr>
<th>Group</th>
<th>Time to occlusion (min)</th>
<th>Incidence of reperfusion (reperfused/total)</th>
<th>Time to reperfusion (min)</th>
<th>Incidence of reocclusion (reocluded/reperfused)</th>
<th>Time to reocclusion (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (t-PA)</td>
<td>45.1±5.2</td>
<td>6/10 (60%)</td>
<td>78.2±5.6</td>
<td>5/6 (83%)</td>
<td>8.0±6.0</td>
</tr>
<tr>
<td>2 (t-PA/Hep)</td>
<td>69.9±4.8</td>
<td>9/10 (90%)</td>
<td>61.9±9.1</td>
<td>8/9 (89%)</td>
<td>10.3±3.1</td>
</tr>
<tr>
<td>3 (t-PA/Hep/Bitis)</td>
<td>58.4±8.0</td>
<td>9/10 (90%)</td>
<td>47.3±7.6*</td>
<td>2/9 (22%)**</td>
<td>1.0, 16.0</td>
</tr>
<tr>
<td>4 (t-PA/Bitis)</td>
<td>58.6±8.4</td>
<td>6/10 (60%)</td>
<td>51.8±8.5</td>
<td>5/6 (83%)</td>
<td>43.0±14.5</td>
</tr>
<tr>
<td>5 (No treatment)</td>
<td>84.4±15.9</td>
<td>0/5 (0%)</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM.

- t-PA, tissue-type plasminogen activator; Hep, heparin; Bitis, bitistatin.
- Groups 1–4 had 10 dogs in each group; group 5 had 5 dogs.
- *p<0.05 compared with Group 1 by analysis of variance followed by Dunnett’s test.
- **p<0.05 compared with all groups by analysis of variance followed by Dunnett’s test.
- †Reocclusion is assessed only in dogs that reperfused in response to t-PA.
t-PA–induced reperfusion in this study was determined by restoration of coronary blood flow in the previously totally occluded coronary artery. For reperfusion to be recorded as such, the magnitude of blood flow restoration required was at least 50% of control blood flow that persisted in that artery for at least 5 minutes. t-PA–induced reperfusion occurred in group 1 dogs at 78.2±5.6 minutes (Table 1). Treatment with heparin did not significantly decrease the lysis time (61.9±9.1 minutes); however, treatment with heparin and bitistatin (Figure 4) significantly decreased t-PA–induced lysis time at 47.3±7.6 minutes (p<0.05 versus group 1). The addition of bitistatin alone (Figure 4) to t-PA (group 4) also decreased lysis time; however, the decrease was not statistically significant. Thus, the reduction in lysis time appears to be related to the addition of heparin and bitistatin to the experimental protocol.

Acute thrombotic reocclusion was assessed in this study for 2 hours after termination of the t-PA infusion. Reocclusion is defined as zero blood flow after successful t-PA–induced reperfusion. The incidence of reocclusion in group 1 t-PA–treated animals was 83%; reocclusion occurred at 8±6 minutes (Table 1). In group 2, which received t-PA and heparin, and group 4, which received t-PA and bitistatin, the overall incidence of reocclusion was similar to group 1. However, in group 3, which received t-PA, heparin, and bitistatin, acute reocclusion occurred in only two of nine (22%) reperfused dogs (p<0.05) (Table 1). Thus, the combination of t-PA, heparin, and bitistatin was an extremely effective regimen for prevention of thrombotic reocclusion in this model.

Platelet aggregation was assessed ex vivo in response to ADP (2.5, 5.0, and 10 μM) throughout the experimental protocol in all groups of dogs (Table 2). In group 1, t-PA slightly enhanced ADP-induced aggregation (10 μM), whereas in group 2 heparin-treated dogs, platelet aggregation was relatively unchanged. However, in groups 3 and 4, treated with bitistatin, platelet aggregatory responses to all concentrations of ADP tested were essentially eliminated (Table 2). Platelet aggregation to collagen and the endoperoxide/thromboxane A2 mimetic U46619, as well as to ADP, was also assessed in some experiments before and after administration of bitistatin. Platelet aggregation to collagen and U46619, as well as ADP, was blocked by bitistatin at a bolus dose of 30 μg/kg i.v. followed by an infusion of 3 μg/kg/min (Figure 5).

Gum bleeding times were also assessed in all groups of dogs studied. Normal (control) gum bleeding time in the dog is 1–2 minutes (Table 3). Dogs that were administered bitistatin (groups 3 and 4) had a 3.5–4.0-fold increase in bleeding time over control, whereas dogs in groups 1, 2, and 5 had no significant change in bleeding time.

To monitor heparin therapy in groups 2 and 3, activated partial thromboplastin time was determined. The control activated partial thromboplastin time for dogs is 10–11 seconds. Heparin at an initial bolus dose of 100 units/kg i.v. followed by a bolus of 50 units/kg every hour thereafter increased activated partial thromboplastin time 1.5–2.0 times over control (data not shown).

Residual clot wet weights were obtained at the end of each experimental protocol. Dogs that received no treatment (no t-PA, heparin, or bitistatin [group 5])

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**Table 2. Ex Vivo Platelet Aggregation to 10 μM ADP in Canine Model of Coronary Thrombosis**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>I (μL)</th>
<th>II (μL)</th>
<th>III (μL)</th>
<th>IV (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (t-PA)</td>
<td>38.6±5.7</td>
<td>45.0±2.2</td>
<td>46.2±3.2</td>
<td>42.8±2.8</td>
</tr>
<tr>
<td>2 (t-PA+Hep)</td>
<td>43.4±9.7</td>
<td>38.9±5.4</td>
<td>53.8±6.1</td>
<td>39.2±4.8</td>
</tr>
<tr>
<td>3 (t-PA+Hep+Bitis)</td>
<td>42.8±7.9</td>
<td>8.2±2.9*</td>
<td>4.4±2.4*</td>
<td>0.3±0.3*</td>
</tr>
<tr>
<td>4 (t-PA+Bitis)</td>
<td>47.0±5.1</td>
<td>4.4±1.6*</td>
<td>3.2±0.7*</td>
<td>2.0±0.0*</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. LTU, light transmission units; I, blood sample taken at occlusion; II, blood sample taken after 30 minutes of tissue-type plasminogen activator (t-PA) administration; III, blood sample taken after 60 minutes of t-PA administration; IV, blood sample taken 1 hour after termination of t-PA; Hep, heparin; Bitis, bitistatin. n=5 dogs for each group. *p<0.05 compared with sample I by analysis of variance followed by Dunnett’s test.
FIGURE 5. Tracings showing effect of bitistatin (30 μg/kg bolus plus 3 μg/kg/min i.v. continuously) on platelet aggregation induced by ADP, U46619, and collagen (sample III in protocol [Figure 3]). At the dose of bitistatin that was used, platelet aggregation is essentially eliminated; however, platelet activation as determined by platelet shape change (downward deflection in the tracing) is unaffected. LTU, light transmission units.

had a thrombus wet weight of 16.5±2.2 mg (Table 4). Dogs in group 1 (t-PA), group 2 (t-PA plus heparin), and group 4 (t-PA plus bitistatin) had residual thrombus masses of approximately 5.0 mg. Only dogs treated with the combination of t-PA, heparin, and bitistatin (group 3) had a significant decrease in thrombus mass to 2.0±0.4 mg.

Fibrinogen levels in dogs before infusion of t-PA averaged approximately 160 mg/dl (Table 5). Infusion of t-PA in groups 1–4 decreased plasma fibrinogen significantly, as expected (Table 5), indicating systemic fibrinogenolysis and an associated lytic state. Dogs in group 5, not administered t-PA, demonstrated no significant fall in plasma fibrinogen. One hour after termination of the t-PA infusion, fibrinogen levels were beginning to return toward control preinfusion levels.

Hemodynamically, dogs in groups 1 and 2 were relatively stable throughout the experimental protocol (Figure 6). Mean arterial blood pressure decreased over the time course of the experiment, an effect that is probably related to the length of the procedure (~8 hours). However, animals administered bitistatin (groups 3 and 4) had a decrease in mean arterial blood pressure (Figure 6) that was related to the fact that these open-chest dogs had many opportunistic sites for bleeding to occur. Thus, in dogs administered the antiplatelet agent bitistatin, blood pressure decreased as a consequence of impaired platelet-dependent hemostatic plug formation. This effect only occurred toward the end of the experimental protocol and was not apparent at reperfusion. Because volume replacement with canine donor whole blood restored blood pressure, the decrease in blood pressure noted in dogs treated with heparin and bitistatin appeared to be related almost entirely to volume loss (data not presented). Furthermore, anesthetized dogs were administered heparin, bitistatin, and t-PA in which no surgery other than placement of intravenous and intra-arterial catheters had been performed. In these dogs, there was little or no decrease in blood pressure as opposed to open-chest, operated dogs receiving heparin, bitistatin, and t-PA.

### Table 3. Bleeding Times in Canine Model of Coronary Thrombosis

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Bleeding time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>1 (t-PA)</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>2 (t-PA+Hep)</td>
<td>1.7±0.1</td>
</tr>
<tr>
<td>3 (t-PA+Hep+Bitis)</td>
<td>1.7±0.1</td>
</tr>
<tr>
<td>4 (t-PA+Bitis)</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>5 (no treatment)</td>
<td>1.6±0.8</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

I, blood sample taken at occlusion; II, blood sample taken after 30 minutes of tissue-type plasminogen activator (t-PA) administration; III, blood sample taken after 60 minutes of t-PA administration; IV, blood sample taken 1 hour after termination of t-PA; Hep, heparin; Bitis, bitistatin. n=10 dogs for groups 1–4; n=5 dogs for group 5.

*p<0.05 compared with control (sample I) by analysis of variance followed by Dunnett’s test.

### Table 4. Residual Clot Wet Weights in Canine Model of Coronary Thrombosis

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (t-PA)</td>
<td>5.0±0.8</td>
</tr>
<tr>
<td>2 (t-PA+Hep)</td>
<td>5.8±1.0</td>
</tr>
<tr>
<td>3 (t-PA+Hep+Bitis)</td>
<td>2.0±0.4*</td>
</tr>
<tr>
<td>4 (t-PA+Bitis)</td>
<td>5.4±1.1</td>
</tr>
<tr>
<td>5 (no treatment)</td>
<td>16.5±0.5</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
t-PA, tissue-type plasminogen activator; Hep, heparin; Bitis, bitistatin. n=10 dogs for groups 1–4; n=5 dogs for group 5.

*p<0.05 compared with all groups.
TABLE 5. Plasma Fibrinogen Levels in Canine Model of Coronary Thrombosis

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Plasma fibrinogen level (mg/dl)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>1 (t-PA)</td>
<td>165.5±6.1</td>
<td>100.8±15.5*</td>
<td>81.5±13.3*</td>
</tr>
<tr>
<td>2 (t-PA+Hep)</td>
<td>155.5±5.0</td>
<td>107.3±21.3*</td>
<td>102.2±14.8*</td>
</tr>
<tr>
<td>3 (t-PA+Hep+Bitis)</td>
<td>157.0±7.2</td>
<td>107.0±14.9*</td>
<td>98.9±15.7*</td>
</tr>
<tr>
<td>4 (t-PA+Bitis)</td>
<td>160.0±13.1</td>
<td>72.3±7.5*</td>
<td>63.6±10.4*</td>
</tr>
<tr>
<td>5 (no treatment)</td>
<td>152.0±11.0</td>
<td>152.0±7.2</td>
<td>158.0±17.0</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

I, blood sample taken at occlusion; II, blood sample taken after 30 minutes of tissue-type plasminogen activator (t-PA) administration; III, blood sample taken after 60 minutes of t-PA administration; IV, blood sample taken 1 hour after termination of t-PA; Hep, heparin; Bitis, bitistatin. n=10 dogs for groups 1–4; n=5 dogs for group 5.

*p<0.05 compared with sample I by analysis of variance followed by Dunnett's test.

Discussion

This study was designed to determine the influence of the RGD-containing peptide bitistatin alone or in combination with heparin on t-PA–induced thrombolysis and subsequent reocclusion. The results of this study indicate that treatment of dogs with the combination of t-PA, heparin, and bitistatin increases the overall incidence of t-PA–induced reperfusion, decreases thrombolysis time significantly, and lowers the incidence of thrombotic reocclusion. Administration of t-PA and heparin did not significantly affect lysis time or reocclusion, whereas t-PA and bitistatin did tend to decrease lysis time but did not influence the incidence of reocclusion. Bitistatin did tend to lengthen the time to acute reocclusion from 8±6 to 43±15 minutes; however, this increase in time to reocclusion was not statistically significant. Thus, from these data, it appears that to decrease lysis time as well as prevent reocclusion both heparin and bitistatin are required in combination with t-PA in this canine model of coronary arterial thrombosis. These pharmacologic data would suggest that the reocclusion observed in this model is due to a combination of new fibrin formation as well as platelet aggregation. Thus, by inhibiting new fibrin formation with heparin and platelet aggregation with bitistatin, the overall efficiency of t-PA–induced thrombolysis was dramatically improved.

Fibrinogen receptor antagonism represents a unique approach to inhibition of platelet aggregation. On platelet activation with various agonists (i.e., ADP, collagen, epinephrine, thrombin, 5-hydroxytryptamine, or thromboxane A2), platelets change shape and then bind plasma fibrinogen by means of the platelet glycoprotein IIb/IIIa receptor complex.14–16 RGD acid, which occurs twice in the Aα chain of fibrinogen, is believed to mediate, at least in part, the binding of fibrinogen to the glycoprotein IIb/IIIa complex.32,33 Thus, binding of fibrinogen to activated platelets is a key event in platelet-to-platelet interaction and plays a major role in thrombus formation.34,35 Fibrinogen receptor antagonists, such as bitistatin, the RGD-containing peptide used in this study, thereby block platelet aggregation independent of the nature of the platelet agonist. This represents a unique mechanism; other antithrombotic approaches have generally been targeted against individual mediators of platelet aggregation rather than on the final common pathway of platelet aggregation (i.e., binding of plasma fibrinogen). Therefore, in situations in which the exact nature of the mediator is unknown, as is the case in most clinical thromboembolic diseases, fibrinogen receptor antagonists should be effective antiplatelet agents.

Figure 6. Bar graphs of mean systemic arterial blood pressure in dogs receiving tissue-type plasminogen activator (t-PA) alone, t-PA plus heparin, t-PA plus heparin plus bitistatin, and t-PA plus bitistatin. Means are given at control (before current initiation), occlusion, t-PA–induced reperfusion, and termination. Dogs treated with bitistatin had a significant decrease in blood pressure as a consequence of bleeding from open-chest wounds.
Previous experimental studies in dogs19-21 and baboons22 have demonstrated the beneficial actions of monoclonal antibodies against platelet glycoprotein IIb/IIIa. These antibodies, termed 7E3 and LJ-CP8, are potent antithrombotic agents in their own right and also influence thrombolysis in a positive manner. However, unlike the RGD-containing fibrinogen receptor antagonist peptides, the effects of these antibodies are essentially irreversible and last for the 7–10-day life span of the platelet.23 We have previously demonstrated29 that bitistatin exhibits dose-dependent inhibition of cyclical flow reduction in the Folts model as well as inhibition of ex vivo platelet aggregation and prolongation of the bleeding time. At the maximal effective inhibitory dose that was used (100 µg/kg i.v. bolus), platelet aggregation and bleeding time were inhibited for 3 hours, at which time values returned to control levels. Thus, the antithrombotic effects of bitistatin are dose-dependent and reversible; thus, careful clinical titration of dosage and control of potential bleeding is possible.

To profoundly affect acute reocclusion in this study, the combination of bitistatin and heparin was required. Neither heparin alone nor bitistatin alone prevented reocclusive events. Controversy currently exists over the use of heparin in combination with thrombolytic agents. In experimental animal studies, heparin has been demonstrated to be beneficial in combination with streptokinase36 as well as t-PA.37,38 The benefit of heparin in these studies was reflected as an enhancement of reperfusion, leading to a greater frequency and more complete state of blood flow restoration. However, a recent clinical study39 has examined 70 patients treated with t-PA alone versus 64 patients treated with 10,000 units of heparin and t-PA. Heparin did not improve vessel patency at 90 minutes after the treatment as determined angiographically (79% for both groups). Thus, these authors concluded that heparin therapy could be deferred for at least 60–90 minutes after the initiation of t-PA. Further clinical studies will be required to determine the chronic effects of anticoagulation and antiplatelet therapy on reocclusion and recurrent ischemia.

The results of this study may have important implications related to the role of adjunctive pharmacologic agents to thrombolytic therapy. Previous investigations have emphasized the role of thromboxane A2 as a mediator of platelet activation in acute myocardial infarction patients receiving streptokinase40 or in experimental animals treated with t-PA or streptokinase.10–12 Advances in platelet physiology have now led to the development of broad-based fibrinogen-receptor antagonists that block platelet aggregation independent of the agonist and do so without any hemodynamic changes. Thus, RGD-containing peptides that function as fibrinogen-receptor antagonists may play a significant and important role as potential adjuncts to thrombolytic therapy. Clot dissolution by fibrinolytic agents appears to be a dynamic process in which new fibrin formation may occur at the site of the residual stenosis, followed by platelet aggregation. Using heparin to interrupt new fibrin formation and using agents similar to bitistatin to inhibit platelet-dependent reocclusion may lead to an improvement in the overall efficiency of thrombolytic therapy. A concern in such an aggressive approach to acute myocardial infarction involves potential bleeding, which will require careful monitoring and control. The potent, yet reversible, effects of RGD-containing peptides on platelet function and subsequent hemostatic plug formation should allow for clinical evaluation of fibrinogen receptor antagonists as an adjunctive approach to thrombolytic therapy.

Acknowledgments

The authors wish to thank Mrs. Robin Carter for preparation of this manuscript. We would also like to acknowledge the assistance of Mr. Timothy Schofield in statistical analysis of the data.

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KEY WORDS: thrombolysis, tissue-type plasminogen activator, reperfusion, bitistatin, heparin.
Acceleration of recombinant tissue-type plasminogen activator-induced thrombolysis and prevention of reocclusion by the combination of heparin and the Arg-Gly-Asp-containing peptide bitistatin in a canine model of coronary thrombosis.

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_Circulation_. 1990;82:169-177
doi: 10.1161/01.CIR.82.1.169

_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

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