In Vivo Thrombin Inhibition Enhances and Sustains Arterial Recanalization With Recombinant Tissue-type Plasminogen Activator

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The effects of heparin and the synthetic competitive thrombin inhibitor \(2R,4R\)-4-methyl-\(\text{N}^2\)-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-\(\text{l}\)-arginy]-2-piperidinecarboxylic acid monohydrate (Argatroban) on thrombolysis with recombinant tissue-type plasminogen activator (rt-PA) was studied in groups of six or seven rabbits with arterial thrombosis. The model consisted of a whole-blood clot produced in a 1-cm isolated femoral arterial segment with superimposed endothelial damage and distal high-grade stenosis. rt-PA was injected as an intravenous bolus of 0.45 mg/kg body wt at 15-minute intervals until recanalization, or up to a maximum of four boluses. In seven rabbits given an intravenous injection of 17 mg/kg heparin, rt-PA induced transient reflow in only one animal. In seven rabbits that received intravenous heparin (200 units/kg over 60 minutes), rt-PA administration produced reflow in five animals, which was persistent in two rabbits. Combined administration of aspirin and heparin in seven rabbits was associated with similar rt-PA–induced recanalization. rt-PA administration in six rabbits given intravenous Argatroban (100 µg/kg/min for 60 minutes) caused recanalization in five, with persistent patency in three. In six rabbits given aspirin and Argatroban, rt-PA caused recanalization in all, with persistent patency in five animals. Reflow occurred significantly more rapidly with Argatroban (14±7 minutes) than with heparin (35±11 minutes), reflow was obtained with fewer boluses of rt-PA in combination with Argatroban (median value of one bolus) than with heparin (median value, three boluses), and reocclusion after reflow was less frequent with Argatroban (0 of 11 versus 5 of 10 rabbits). Furthermore, the degree of thrombolysis determined by pathological analysis was significantly more extensive with Argatroban than with heparin, and patency persisted during a 3-hour observation period, despite elimination of Argatroban from the circulation. Thus, Argatroban, relative to heparin, enhances and sustains thrombolysis with rt-PA. It may offer promise as an adjunctive agent for thrombolytic therapy of arterial thrombosis. (*Circulation Research* 1990;67:1552–1561)

Thrombolytic therapy has become an established treatment for selected patients with acute transmural myocardial infarction. However, currently used agents and administration schemes still suffer significant shortcomings including 1) resistance to pharmacological recanalization in up to 25% of patients, even with the most potent agent; 2) delayed onset of reflow, averaging 45 minutes in the setting of progressive myocardial necrosis; 3) reocclusion after initial successful recanalization, occurring in 5–30% of initially reperfused patients; and 4) occurrence of major bleeding in up to 5% of patients in the absence of cardiac interventions.

To overcome these difficulties, a number of experimental approaches to accelerate reperfusion and prevent reocclusion are being explored. These include the use of anticoagulants to prevent additional fibrin deposition and of prostaglandin E and combinations of thromboxane A and serotonin receptor antagonists to prevent continued platelet activation. Furthermore, a monoclonal antibody

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against the platelet glycoprotein IIb/IIIa has been shown to accelerate coronary thrombolysis and to prevent reocclusion in dogs, but its use at an effective dose is associated with a marked prolongation of the bleeding time. Selective inhibition of thrombin constitutes another effective approach for the prevention of platelet-rich thrombus formation and the acceleration of clot lysis.

In the present study, we investigated the effect of the synthetic thrombin inhibitor (2R,4R)-4-methyl-1-[N²-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-L-arginyl]-2-piperidinecarboxylic acid monohydrate (Argatroban) relative to heparin, used alone or in combination with aspirin, on the degree and stability of recanalization with recombinant tissue-type plasminogen activator (rt-PA). A rabbit femoral arterial thrombosis model with superimposed endothelial damage and high-grade stenosis was used. This model is resistant to lysis with rt-PA in the absence of anticoagulation and has a high frequency of reocclusion after initial recanalization. Our results confirm that Argatroban, relative to heparin, accelerates clot lysis and prevents reocclusion. In addition, our results indicate that recanalization requires less rt-PA, that the degree of clot lysis is higher, and that the stability of recanalization is better in the groups receiving Argatroban than in the groups with heparin when used at a comparable anticoagulant dose. Stable recanalization persists despite elimination of the drug from plasma and normalization of the bleeding time.

Materials and Methods

Reagents

The synthetic thrombin inhibitor Argatroban and rt-PA (Activase) were supplied by Genentech, Inc., South San Francisco, Calif. Argatroban was supplied in a ready-to-use solution at a concentration of 0.5 mg/ml. rt-PA was supplied as a powder to be dissolved in sterile water to a concentration of 1 mg/ml. Heparin was purchased from Elkins-Sinn, Inc., Cherry Hill, N.J. Aspirin for intravenous administration was obtained from Synthelabo Benelux, Brussels, in vials containing 900 mg lysine acetylsalicylic acid (equivalent to 500 mg acetylsalicylic acid per vial), which was dissolved in sterile water before use. The biochemical and biological properties of Argatroban have been reported elsewhere.

Rabbit Femoral Arterial Thrombosis Model

The femoral arterial thrombosis model with superimposed endothelial cell damage and high-grade stenosis is schematically represented in Figure 1. New Zealand White rabbits of either sex, weighing from 2.2 to 4.0 kg, were anesthetized with an intravenous injection of sodium pentobarbital (35 mg/kg followed by 10-mg boluses when needed) via a marginal ear vein. The right brachial artery was cannulated under a surgical microscope (Wild M651, Heerbrugg, Switzerland) with an Intracath cannula (22 gauge, Deseret Medical Inc., Becton Dickinson Co., Sandy, Utah), and the right brachial vein and right femoral vein were cannulated with Silastic tubing (i.d. = 0.020 in., Dow Corning Corp., Midland, Mich.).

The left femoral artery was then exposed between the inguinal ligament and the distal bifurcation. The superficial epigastric artery was cannulated with an Intracath cannula (22 gauge). The baseline blood flow in the left femoral artery was measured with an ultrasonic flowmeter (T101 Transonic System Inc., Ithaca, N.Y.). Simultaneously, blood pressure was measured in the brachial artery. The stenosis was produced proximal to the flow probe by stepwise constriction of the artery with two 3-0 Vicryl sutures (Ethicon Inc., Somerville, N.J.) to reduce blood flow to approximately 50% of baseline. To allow the flow to be accurately adjusted after introduction of the sutures, five 4-mm-long Vicryl threads were inserted under the sutures parallel to the longitudinal axis of the vessel. These threads were removed one by one until the poststenotic flow reached 50±10% of the baseline flow.

Snare occlusions were made proximal and distal to the cannulated epigastric artery, and a 1-cm-long isolated vessel segment of the femoral artery was emptied of blood. The isolated segment was traumatized by repeated external compression with blunt forceps to produce endothelial injury. Thrombin (0.05 ml Thrombin, Armour Pharmaceutical, Kankakee, Ill.) and freshly drawn blood (0.1 ml) were injected through the epigastric artery cannula. Then, the proximal snare was released followed 10 minutes later by release of the distal snare. The absence of blood flow was monitored for 10 minutes with the flow probe to document stable occlusion.

Infusion Protocols and Evaluation of Femoral Arterial Patency

The following five groups of six or seven rabbits were studied: 1) intravenous bolus injection of 30

![Figure 1. Schematic illustration of the femoral arterial thrombosis model with superimposed endothelial cell damage and high-grade stenosis. RBC, red blood cell.](image-url)
mg/kg lysine acetylsalicylic acid (equivalent to 17 mg/kg aspirin). 2) intravenous infusion of 200 units/kg heparin over 60 minutes, 3) intravenous administration of the combination of heparin and aspirin, 4) intravenous infusion of 100 μg/kg/min Argatroban over 60 minutes, and 5) administration of the combination of aspirin and Argatroban. The study was carried out in two phases. Initially, animals were assigned at random to the rt-PA+heparin, rt-PA+Argatroban, or rt-PA+aspirin+Argatroban groups. Toward the end of this phase, the rt-PA+aspirin and the rt-PA+aspirin+heparin groups were added. However, as indicated in the results section, the model was highly reproducible, as judged from the very similar values for baseline blood flow, poststenotic blood flow, and the blood flow in animals with persistent patency (see Table 1).

The intravenous administration was given via the right femoral vein. The infusion was carried out with a constant rate infusion pump (syringe infusion pump 22, Harvard Apparatus, South Natick, Mass.). Ten minutes after the start of the administration of these agents, rt-PA was given via the brachial vein in a bolus of 0.45 mg/kg at 15-minute intervals until recanalization of the thrombosed femoral artery was achieved, or up to a maximum of four boluses. Blood flow was recorded continuously with the ultrasonic blood flow probe throughout the experimental period. Reflow was prospectively defined as a blood flow of more than 50% of the poststenotic flow (more than 25% of baseline flow). The recanalization time was taken as the time from the first injection of rt-PA until reflow was documented. No additional rt-PA was given once recanalization was achieved, even if reocclusion followed. Occlusion was prospectively defined as blood flow less than 15% of the poststenotic flow (approximately 0.8 ml/min), at which time the phasic pattern of blood flow could no longer be observed. Partial reflow was defined as a blood flow between 15% and 50% of the poststenotic flow. Femoral arterial patency status was categorized as follows: 1) persistent occlusion, no significant blood flow (<0.8 ml/min) through the artery throughout the experiment; 2) reocclusion after reflow, reocclusion persisting for at least 60 minutes before the end of the experiment, after initial reflow; 3) cyclic reflow, alternating reocclusion and recanalization after initial reflow; 4) persistent patency, persistent flow without reocclusion after initial reflow. The studies with experimental animals were carried out conforming to the guiding principles of the American Physiological Society.

**Hemostasis Assays**

Blood samples were taken at baseline and 50 minutes and 2 and 4 hours after the start of the administration of aspirin, heparin, or Argatroban. Blood samples for determination of the activated partial thromboplastin time and thrombin time were drawn into 0.01 M citrate. The partial thromboplastin time was performed in a fibrometer with 0.1 ml citrated plasma and 0.1 ml Kontact reagent (Pacific Hemostasis, Ventura, Calif.) to which 0.1 ml CaCl₂ solution was added after 5 minutes of incubation at 37°C. Normal values for rabbits ranged between 15 and 25 seconds when experiments were performed with fresh frozen plasma. The thrombin time was determined in a fibrometer with 0.2 ml citrated plasma and 0.1 ml Thrombinex solution (Bio/Data, Huttboro, Pa.). Normal values for rabbits range between 15 and 30 seconds.

Samples for rt-PA and fibrinogen determination were collected in 0.01 M citrate and aprotinin (150 kallikrein inhibitor units/ml, Sigma Chemical Co., St. Louis). Fibrinogen was measured by the coagulation rate assay of Clauss as modified by Vermyle et al. This assay is insensitive to heparin concentrations in plasma up to 10 units/ml, rt-PA antigen was measured by enzyme-linked immunosorbent assay as described elsewhere. Six rabbits treated with aspirin were randomly chosen, and platelet aggregation with 1 μl epinephrine (0.5 μM) and 14 μl arachidonic acid (0.5 μM) per 250 μl platelet-rich plasma was performed.

Template bleeding times were performed on the shaved left medial foreleg with a spring-loaded blade device (Surgicutt International Technidyne Corp., Edison, N.J.).

**Pathological Examination**

At the end of the experiment, all rabbits were killed with an overdose of pentobarbital for pathological examination of the femoral arteries. Segments of the femoral artery including the stenosis were removed and fixed overnight in 5% formaldehyde. The segments were embedded in paraffin blocks and sectioned longitudinally, stained with hematoxylin and cosin, and evaluated microscopically. Some persistently patent arteries were subjected to perfusion-fixation with 0.1 M cacodylate buffered with 2.5% glutaraldehyde for scanning electron microscopy as previously described.

The extent of thrombosis was semiquantitatively graded on a scale of 1–4 with 1, no or minimal mural thrombus; 2, mural thrombus occupying less than 50% of the luminal diameter throughout the length of the isolated arterial segment; 3, thrombus occupying more than 50% but less than 95% of the luminal diameter; and 4, completely occlusive or greater than 95% luminal thrombus. The composition of the thrombus was characterized as erythrocyte-rich, platelet-rich, or mixed with interlaced platelet-rich and erythrocyte-rich zones.

**Statistical Analysis**

The values are expressed as mean±SD. The significance of differences between groups was determined with Student’s t test for paired or unpaired values. A Kruskal-Wallis nonparametric analysis of variance was performed on ranks of the ordered variable of arterial patency, which ranges from 0 to 3 (0, persistent occlusion; 1, reflow with reocclusion; 2,
cyclic reflow and reocclusion; 3, persistent patency as
determined with the blood flowmeter). A similar
analysis was performed on arterial patency graded on
pathological analysis as described above. This form of
analysis of variance was selected because of the non-
Gaussian distribution of the patency-state variables.
Fisher’s exact test was used to compare the occurrence
of reflow and reocclusion in the various groups.

Results

Effect of Aspirin, Heparin, and Argatroban on
Femoral Artery Recanalization With Recombinant
Tissue-type Plasminogen Activator

Results of continuous femoral arterial blood flow
measurements are shown in Table 1. The baseline
blood flow in the femoral artery in the various groups
ranged between 10 and 13 ml/min. The external
constriction reduced the blood flow to 50±7% of
baseline. The results of blood flow measurements
between groups before and after constriction were
indistinguishable, which is indicative of the reproduc-
ibility of the model and its stability in time. At the
end of the experiment (4 hours), the mean blood flow
in the groups treated with Argatroban was signifi-
cantly higher than that in the groups with heparin
(26±21% versus 7.7±14%, p=0.02). This mainly
reflected a difference in frequency of reocclusion,
because blood flow was not significantly different in
rabbits with persistent patency.

The results of the evaluation of the femoral arte-
rnal patency with the five different infusion protocols,
categorized as persistent occlusion, reocclusion after
initial reflow, cyclic reflow and reocclusion, and
persistent patency, as defined in “Materials and
Methods,” are summarized in Table 2. The time
course of femoral artery recanalization in the individual
animals is schematically represented in Figure
2. Injection of four boluses of rt-PA in animals given
intravenous aspirin induced recanalization in one of
seven animals only as illustrated in the figure (experi-
ment 1). Reflow was achieved after 47 minutes, and
after three cyclic periods, the vessel remained oc-
ccluded. rt-PA combined with heparin-induced reflow
in five of seven rabbits (experiments 1, 4, 5, 6, and 7),
with a time to recanalization of 37±9 minutes
(mean±SD). In two of these four rabbits (experi-
ments 4 and 6) the artery reoccluded after a short
cyclic period, in two rabbits (experiments 5 and 7) it
remained patent, and in the other one (experiment 1)
it demonstrated cyclic changes throughout the exper-
iment. The median value of the number of rt-PA
boluses was three, with a range of two to four (Table
2). The group treated with aspirin and heparin in
combination with rt-PA showed recanalization in five
of seven rabbits (experiments 1, 3, 4, 5, and 6) with
an average time to reflow of 33±13 minutes. Reocclu-
sion occurred in three of these five animals (experi-
ments 1, 4, and 5), while persistent patency was
observed in the other two (experiments 3 and 6). A
median of three rt-PA boluses was required, with a
range of two to four. In the group treated with
Argatroban and rt-PA, recanalization was achieved
in five of six animals (experiments 1, 2, 4, 5, and 6),
with a time to reflow of 12±7 minutes, and reocclu-

<table>
<thead>
<tr>
<th>Infusion protocol</th>
<th>Baseline (ml/min)</th>
<th>After constriction</th>
<th>All</th>
<th>Persistent patency</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt-PA+ASA</td>
<td>11±5.5</td>
<td>51±9</td>
<td>0.3±0.7</td>
<td>...</td>
</tr>
<tr>
<td>rt-PA+Hep</td>
<td>10±3.2</td>
<td>48±9</td>
<td>7.4±16</td>
<td>41 (2)</td>
</tr>
<tr>
<td>rt-PA+ASA+Hep</td>
<td>11±3.2</td>
<td>50±5</td>
<td>8.1±13</td>
<td>24 (2)</td>
</tr>
<tr>
<td>rt-PA+Arg</td>
<td>10±2.7</td>
<td>52±4</td>
<td>21±14</td>
<td>33±6 (3)</td>
</tr>
<tr>
<td>rt-PA+ASA+Arg</td>
<td>13±4.6</td>
<td>46±5</td>
<td>32±27</td>
<td>37±26 (5)</td>
</tr>
</tbody>
</table>

Data represent mean±SD, expressed in percent of baseline, with number of animals in parentheses. rt-PA, recombinant tissue-type plasminogen activator; ASA, aspirin; Hep, heparin; Arg, Argatroban.

<table>
<thead>
<tr>
<th>Infusion protocol</th>
<th>n</th>
<th>Patency status*</th>
<th>Time to reflow (min)</th>
<th>Number of rt-PA boluses</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt-PA+ASA</td>
<td>7</td>
<td>PO RR CR PP</td>
<td>47</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>rt-PA+Hep</td>
<td>7</td>
<td>2 2 1 2</td>
<td>37±9</td>
<td>3</td>
<td>2–4</td>
<td></td>
</tr>
<tr>
<td>rt-PA+ASA+Hep</td>
<td>7</td>
<td>2 3 0 2</td>
<td>33±13</td>
<td>3</td>
<td>2–4</td>
<td></td>
</tr>
<tr>
<td>rt-PA+Arg</td>
<td>6</td>
<td>1 0 2 3</td>
<td>12±7</td>
<td>1</td>
<td>1–4</td>
<td></td>
</tr>
<tr>
<td>rt-PA+ASA+Arg</td>
<td>6</td>
<td>0 0 1 5</td>
<td>15±8</td>
<td>1.5</td>
<td>1–3</td>
<td></td>
</tr>
</tbody>
</table>

Data represent frequencies and mean±SD. n, Number of animals in the group; rt-PA, recombinant tissue-type plasminogen activator; PO, persistent occlusion; RR, reocclusion after reflow; CR, cyclic reflow; PP, persistent patency; ASA, aspirin; Hep, heparin; Arg, Argatroban.

*p=0.004 for significant differences in patency between experimental groups reported in this table (Kruskal-Wallis nonparametric analysis of variance).
sion was not observed. Two of these five rabbits (experiments 1 and 2) showed cyclic reflow, and the other three animals (experiments 4, 5, and 6) showed persistent patency. The median values of the number of rt-PA boluses was one, with a range of one to four. All six rabbits treated with aspirin and Argatroban in combination with rt-PA showed recanalization within 15±8 minutes. In this group, five rabbits had persistent patency, and one animal (experiment 3) showed cyclic reflow. The median number of rt-PA boluses given was 1.5, with a range of one to three.

Several significant differences in femoral arterial patency were observed with the various infusion protocols. The time to reflow was significantly shorter with Argatroban than with heparin, both in the absence of aspirin (12±7 versus 37±9 minutes, p=0.001) and in the presence of aspirin (15±8 versus 33±13 minutes, p=0.02), although the frequency of reperfusion (10 of 13 versus 11 of 13 rabbits) was not significantly different. The addition of aspirin did not significantly influence the time to reperfusion with either heparin (37±9 versus 33±13 minutes) or Argatroban (12±7 versus 15±8 minutes).

A Kruskal-Wallis analysis of the patency status of all experiments yielded a value of p=0.004. The femoral arterial patency status was significantly better (p=0.04) in rabbits receiving Argatroban (no reocclusion, three with cyclic reflow and eight with persistent patency) than in rabbits receiving heparin (five with reocclusion, one with cyclic reflow, and four with persistent patency). A Kruskal-Wallis analysis of the number of rt-PA boluses given to achieve reperfusion yielded a value of p=0.0001 for significant differences between groups, with p=0.032 between rt-PA+heparin and rt-PA+Argatroban and p=0.018 between rt-PA+aspirin+heparin and rt-PA+aspirin+Argatroban.

Hemostasis Analyses

Table 3 summarizes the results of activated partial thromboplastin times, thrombin times, and plasma fibrinogen levels. Aspirin in combination with rt-PA did not alter the activated partial thromboplastin time, whereas the transient prolongation of the thrombin time could be ascribed to fibrinogen degradation with transiently elevated fibrinogen degradation products generated by the four bolus injections of rt-PA. Argatroban, at a dose of 100 μg/kg/min for 60 minutes, prolonged the activated partial thromboplastin time 2.5- to 3.5-fold and the throm-
bin time more than fourfold, whereas heparin at 200 units/kg prolonged both the activated partial thromboplastin time and the thrombin time more than fourfold. At the end of the experiment, the activated partial thromboplastin time had normalized in both groups, but the thrombin time was still prolonged, about threefold, in both groups. These results indicate that the anticoagulant effects of heparin and Argatroban measured at the level of thrombin were very similar but that the effect of heparin on the activated partial thromboplastin time was more pronounced. Platelet aggregation with arachidonic acid and epinephrine was totally abolished in all of six rabbits after intravenous aspirin administration at a dose of 17 mg/kg but was not consistently abolished (only in three of five rabbits) at a dose of 9 mg/kg (data not shown). Therefore, the former dose was used in the present study.

Changes of fibrinogen levels correlated with the number of rt-PA boluses given. In 10 animals that received four boluses of rt-PA, irrespective of the infusion protocol, fibrinogen decreased from 2.5±0.8 to 1.0±0.6 g/l (43±4% of baseline) at the end of the experiment. In four animals given three boluses of rt-PA, the residual fibrinogen level decreased to 66±24%, in six rabbits with two boluses, it decreased to 75±16%, and in eight rabbits receiving one bolus of rt-PA, the residual fibrinogen level at the end of the experiment was 75±5% of baseline.

Several template bleeding times were measured in all animals, with the results summarized in Table 4. Aspirin in combination with rt-PA prolonged the bleeding time from 3.5±0.9 to 12±8.1 minutes, whereas heparin plus rt-PA induced a prolongation from 4.3±0.8 to 5.8±1.1 minutes. However, when these three agents, rt-PA, aspirin, and heparin, were

### Table 3. Activated Partial Thromboplastin Times, Thrombin Times, and Fibrinogen Levels Obtained With the Different Infusion Protocols

<table>
<thead>
<tr>
<th>Infusion protocol</th>
<th>Baseline</th>
<th>50 min</th>
<th>2 hr</th>
<th>4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt-PA + ASA</td>
<td>18±2</td>
<td>19±2</td>
<td>18±2</td>
<td>18±3</td>
</tr>
<tr>
<td>rt-PA + Hep</td>
<td>18±4</td>
<td>&gt;100</td>
<td>71±18</td>
<td>24±7</td>
</tr>
<tr>
<td>rt-PA + ASA + Hep</td>
<td>15±3</td>
<td>98±5</td>
<td>66±20</td>
<td>21±2</td>
</tr>
<tr>
<td>rt-PA + Arg</td>
<td>17±3</td>
<td>60±21</td>
<td>34±13</td>
<td>20±4</td>
</tr>
<tr>
<td>rt-PA + ASA + Arg</td>
<td>21±2</td>
<td>54±6</td>
<td>30±6</td>
<td>21±3</td>
</tr>
</tbody>
</table>

**Thrombin times (sec)**

<table>
<thead>
<tr>
<th>Infusion protocol</th>
<th>Baseline</th>
<th>50 min</th>
<th>2 hr</th>
<th>4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt-PA + ASA</td>
<td>21±1</td>
<td>75±17</td>
<td>32±6</td>
<td>26±3</td>
</tr>
<tr>
<td>rt-PA + Hep</td>
<td>28±2</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>rt-PA + ASA + Hep</td>
<td>27±10</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>rt-PA + Arg</td>
<td>25±7</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>rt-PA + ASA + Arg</td>
<td>22±2</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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</tbody>
</table>

**Fibrinogen (g/l)**

<table>
<thead>
<tr>
<th>Infusion protocol</th>
<th>Baseline</th>
<th>50 min</th>
<th>2 hr</th>
<th>4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt-PA + ASA</td>
<td>2.2±0.4</td>
<td>1.1±0.6</td>
<td>0.6±0.3</td>
<td>0.8±0.3</td>
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<tr>
<td>rt-PA + Hep</td>
<td>2.4±1.4</td>
<td>2.3±1.1</td>
<td>2.1±0.6</td>
<td>1.8±0.5</td>
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<tr>
<td>rt-PA + ASA + Hep</td>
<td>2.3±0.3</td>
<td>1.3±0.3</td>
<td>1.1±0.4</td>
<td>1.1±0.4</td>
</tr>
<tr>
<td>rt-PA + Arg</td>
<td>2.9±1.3</td>
<td>2.3±1.3</td>
<td>2.1±1.0</td>
<td>2.0±0.9</td>
</tr>
<tr>
<td>rt-PA + ASA + Arg</td>
<td>2.1±0.2</td>
<td>1.6±0.3</td>
<td>1.6±0.2</td>
<td>1.5±0.1</td>
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</tbody>
</table>

Data represent mean±SD. Values greater than 100 seconds were given a value of 100 for calculation of means. rt-PA, recombinant tissue-type plasminogen activator; ASA, aspirin; Hep, heparin; Arg, Argatroban.

### Table 4. Template Bleeding Times Obtained With the Different Infusion Protocols

<table>
<thead>
<tr>
<th>Infusion protocol</th>
<th>Baseline</th>
<th>50 min</th>
<th>2 hr</th>
<th>4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt-PA + ASA</td>
<td>3.5±0.9</td>
<td>12±8.1</td>
<td>4.1±1.5</td>
<td>3.3±1.0</td>
</tr>
<tr>
<td>rt-PA + Hep</td>
<td>4.3±0.8</td>
<td>5.8±1.1</td>
<td>4.1±1.2</td>
<td>4.0±0.5</td>
</tr>
<tr>
<td>rt-PA + ASA + Hep</td>
<td>3.1±0.8</td>
<td>20±9.7</td>
<td>4.0±1.3</td>
<td>2.9±1.0</td>
</tr>
<tr>
<td>rt-PA + Arg</td>
<td>3.8±0.9</td>
<td>13±8.2</td>
<td>7.1±7.4</td>
<td>3.6±1.2</td>
</tr>
<tr>
<td>rt-PA + ASA + Arg</td>
<td>4.2±1.2</td>
<td>22±4.7</td>
<td>5.8±1.7</td>
<td>3.6±0.8</td>
</tr>
</tbody>
</table>

Data, expressed in minutes, represent mean±SD. rt-PA, recombinant tissue-type plasminogen activator; ASA, aspirin; Hep, heparin; Arg, Argatroban.
TABLE 5. Results of Pathological Analysis of Femoral Arterial Segments

<table>
<thead>
<tr>
<th>Infusion protocol</th>
<th>Rabbit number</th>
<th>Thrombus grading*</th>
<th>Thrombus description</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt-PA+ASA</td>
<td>1</td>
<td>4</td>
<td>MPE at stenosis</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>ER</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>ER</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>ER</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3</td>
<td>PR</td>
</tr>
<tr>
<td>rt-PA+Hep</td>
<td>1</td>
<td>4</td>
<td>MPE at stenosis</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>MPE</td>
</tr>
<tr>
<td></td>
<td>3</td>
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<tr>
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<td>4</td>
<td>MPE</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1</td>
<td>PR</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4</td>
<td>ER</td>
</tr>
<tr>
<td>rt-PA+ASA+Hep</td>
<td>1</td>
<td>2</td>
<td>MPE</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>ER</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
<td>MPE</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3</td>
<td>MPE†</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>4</td>
<td>ER</td>
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<tr>
<td>rt-PA+Arg</td>
<td>1</td>
<td>4</td>
<td>MPE</td>
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<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>PR</td>
</tr>
<tr>
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<tr>
<td></td>
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</tr>
<tr>
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<tr>
<td></td>
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<td>2</td>
<td>MT; MPE†</td>
</tr>
<tr>
<td>rt-PA+ASA+Arg</td>
<td>1</td>
<td>2</td>
<td>MPE†</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>MPE†</td>
</tr>
<tr>
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<td>1</td>
<td>MPE</td>
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<tr>
<td></td>
<td>4</td>
<td>1</td>
<td>PR</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1</td>
<td>PR</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1</td>
<td>PR†</td>
</tr>
</tbody>
</table>

Thrombus extent was graded from 1 to 4, ranging from minimal mural thrombus (grade 1) to occlusive or subocclusive thrombus (grade 4). Thrombus composition is characterized as MPE, mixed with interlaced platelet-rich and erythrocyte-rich zones; ER, erythrocyte-rich; PR, platelet-rich; rt-PA, recombinant tissue-type plasminogen activator; ASA, aspirin; Hep, heparin; Arg, Argatroban.

*p = 0.01 for significant differences in thrombus grade between experimental groups in this table (Kruskal-Wallis nonparametric analysis of variance).

†Analysis by scanning electron microscopy.

combined, the bleeding time was markedly prolonged, from 3.1±0.8 to 20±9.7 minutes. Argatroban combined with rt-PA prolonged the bleeding time to a level comparable to that obtained with aspirin and rt-PA, from 3.8±0.9 to 13±8.2 minutes. Again, the combination of aspirin and Argatroban with rt-PA markedly prolonged the bleeding time from 4.2±1.2 to 22±4.7 minutes. All bleeding times normalized within 2 hours after the start of the infusion protocols.

Pathology

The results of pathological analysis are summarized in Table 5. The extent of thrombosis was graded, and the thrombus composition was characterized as described in “Materials and Methods” and Table 5. The pathological analysis was performed by an investigator blinded with respect to the infusion protocols. A Kruskal-Wallis analysis of the graded categories of the extent of thrombosis yielded *p = 0.01 for significant overall differences between the groups and *p = 0.01 for the difference between the rt-PA+aspirin+Argatroban and the rt-PA+aspirin+heparin groups.

Segments with persistent occlusion contained occlusive red blood cell clots, segments with reclosure after initial reflow contained occlusive thrombi composed of both red blood cell and platelet-rich clots (Figure 3), whereas segments with persistent patency showed predominantly platelet-rich or mixed mural thrombi. All vessels showed significant mural disruption with intramural hemorrhage. Scanning electron microscopy of persistently patent femoral arterial segments revealed an irregular intimal surface, denuded of endothelium, and areas covered with platelets, neutrophils, and few red blood cells (Figures 4A and 4B). From animals that received Argatroban, fibrin strands were present in only one of three vessels examined by scanning electron microscopy.

Discussion

Various approaches to overcome resistance of coronary artery occlusions to thrombolytic agents, to

FIGURE 3. Light micrograph of a femoral arterial segment from a rabbit treated with heparin and recombinant tissue-type plasminogen activator, which resulted in recanalization followed by reocclusion. The occlusive thrombus in the stenotic segment (arrow) is a platelet-rich clot (P), whereas the proximal vessel is filled with a red blood cell clot (R). Hematoxylin and eosin stain; magnification, ×31.
increase the speed of recanalization, to reduce the frequency of reocclusion, and to minimize the bleeding tendency in association with thrombolytic therapy are currently under investigation in laboratory animal models. One of these approaches consists of the use of synthetic thrombin inhibitors as adjunctive agents.\textsuperscript{23–28} Fitzgerald et al.\textsuperscript{23} have recently demonstrated, in a canine model of electrically induced coronary artery thrombosis, that the synthetic thrombin inhibitor Argatroban accelerates clot lysis by a continuous infusion of rt-PA. However, prevention of reocclusion required a potent thromboxane A\textsubscript{2} inhibitor in addition to a continuous infusion of the thrombin inhibitor.\textsuperscript{28}

In the present study, we have used a rabbit femoral arterial whole-blood clot model with superimposed high-grade stenosis and endothelial damage. In this model, similar to an analogous model in dog coronary artery,\textsuperscript{38} the stenosis markedly increases the resistance to thrombolytic agents and predisposes the artery to reocclusion with platelet-rich material. Our results indicate that Argatroban is significantly more effective than heparin, when infused at a rate that has a similar effect on the thrombin time but a smaller effect on the activated partial thromboplastin time, both with respect to the speed of recanalization and the prevention of reocclusion. These observations extend previous findings with this inhibitor, which included the prevention of arterial occlusion with platelet-rich material,\textsuperscript{26} the abolition of cyclic reflow variations in dogs with coronary artery stenosis and endothelial cell injury,\textsuperscript{39} the acceleration of

FIGURE 4. Scanning electron micrograph of a persistently patent femoral artery segment in a rabbit given two boluses of 0.45 mg/kg recombinant tissue-type plasminogen activator in combination with aspirin and Argatroban. Panel A: Low magnification of the bisected arterial segment without evidence of occlusive thrombus. The site of stenosis is indicated by the arrow. Magnification, ×11. Panel B: High magnification of the intimal surface within the stenotic segment revealing numerous platelets and a few red blood cells (R) but no visible fibrin strands. Magnification, ×1,300.
coronary thrombolysis with rt-PA,23 and the prevention of reocclusion.20 In addition, our study indicates that recanalization occurred with fewer bolus injections in association with Argatroban than with heparin. Both the degree of thrombolysis, as determined by the grading of residual thrombus by using pathological analysis, and the stability of vessel patency monitored up to 3 hours after the end of the infusion of anticoagulant were significantly better in the Argatroban groups than in the heparin groups. These findings are consistent with the hypothesis that thrombin-mediated pathways of platelet activation play a significant role in the pathogenesis of arterial thrombosis.24,26,28,39

It is possible that, at much higher doses, heparin might also have yielded some of the same effects as observed for Argatroban. However, the dose of heparin used in the present study produced activated partial thromboplastin times during the infusion of over 100 seconds in 13 of 14 animals, with a residual fourfold prolongation over baseline 1 hour after the end of the infusion. In humans, such a dose would be considered outside the accepted therapeutic range, especially in association with thrombolytic therapy.

Argatroban in combination with rt-PA prolonged the template bleeding time somewhat more than heparin combined with rt-PA. This bleeding time prolongation was more pronounced in the groups that also received aspirin. However, all bleeding times normalized within 1 hour after the end of the infusion. Moreover, pathological examination in the group receiving Argatroban and aspirin revealed patent arteries without fibrin-rich mural thrombus, despite elimination of the thrombin inhibitor from the circulation. Inasmuch as the template bleeding is a marker of in vivo platelet function,40 the somewhat more pronounced effect of Argatroban as compared with heparin, in the presence of comparable effects on the activated partial thromboplastin time and thrombin time, suggests that Argatroban interferes more effectively with a physiologically relevant thrombin-mediated pathway of platelet activation.

Our findings indicate, at least in this experimental model, that the synthetic thrombin inhibitor Argatroban is very effective with respect to acceleration of arterial recanalization with rt-PA and for the prevention of reocclusion as recently reported by Fitzgerald and FitzGerald.28 In addition, recanalization was more extensive and more stable in the presence of Argatroban and was obtained with less rt-PA. The persistence of patency for up to 3 hours after the end of the infusion, when most of the antithrombin activity of this short-lived inhibitor41 has disappeared from the circulation, suggests that the thrombogenicity of the vessel wall may be transient and overcome by a short-term infusion. Consequently, provided the stability of arterial patency can be confirmed in chronic experiments, infusion of Argatroban in combination with rt-PA may offer promise for improved effective and safe pharmacological recanalization of occluded coronary arteries in patients with acute myocardial infarction.

Acknowledgment

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