Prevention of Platelet-Rich Arterial Thrombosis by Selective Thrombin Inhibition

Ik-Kyung Jang, MD, Herman K. Gold, MD, Andrew A. Ziskind, MD, Robert C. Leinbach, MD, John T. Fallon, MD, PhD, and Désiré Collen, MD, PhD

The effect of heparin and of the synthetic competitive thrombin inhibitor (2R,4R)-4-methyl-1-[N²-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-L-arginy1]-2-piperidinecarboxylic acid monohydrate (argatroban) on platelet-rich arterial thrombosis was studied in a rabbit model, consisting of a 4–6-mm everted (“inside-out”) femoral arterial segment. Intravenous injection of heparin (200 units/kg) failed to prevent occlusion within 60 minutes in all 10 rabbits, whereas intravenous argatroban infusion at a rate of 100 or 200 μg/kg/min for 60 minutes, which prolonged the thrombin time more than fourfold, prevented thrombosis in nine of 13 rabbits (p=0.002 vs. i.v. heparin). Intra-arterial infusion of 200 units/kg heparin over 60 minutes prevented occlusion in six of nine rabbits (p=0.003 vs. i.v. heparin), whereas intra-arterial argatroban at a rate of 100 μg/kg/min for 60 minutes prevented thrombosis in all 10 rabbits (p=0.00001 vs. i.v. heparin). Patency of femoral arterial segments was maintained after the end of the intra-arterial heparin and intravenous or intra-arterial argatroban infusion for up to 3 hours despite normalization of the thrombin time and partial thromboplastin time. Pathologic examination of the graft revealed that the inverted adventitial surface was covered by layers of platelets without platelet aggregation or fibrin deposition. These findings indicate that thrombin plays an important role in platelet-rich arterial thrombosis, and that the thrombogenic stimulus is rapidly attenuated by short-term infusion of the synthetic thrombin inhibitor. Selective thrombin inhibition can constitute an alternative approach to the prevention of arterial occlusion after angioplasty or thrombolytic therapy in patients with unstable coronary syndromes. (Circulation 1990;81:219–225)

Arterial occlusion after successful coronary thrombolysis or angioplasty remains a significant problem in the management of ischemic heart disease.1–4 Coronary occlusion or reclosure at the site of high-grade stenosis or ruptured atheromatous plaque is initiated by platelet-rich deposits5–9 and can occur despite full heparinization.1–4 The primary role of platelets in coronary arterial occlusion has also been documented in patients with coronary artery disease7 as well as in animal models.8–10 The role of thrombin in the pathogenesis of platelet-rich arterial occlusion has been questioned on the basis of limited efficacy of heparin in the prevention of these phenomena.11 Hanson and Harker,12 however, recently reported that a synthetic chloromethyl ketone, which inhibits thrombin efficiently, prevents platelet deposition in arterial dacron grafts, whereas Heras et al13 reported that recombinant hirudin prevents platelet-fibrin deposition during angioplasty in pigs.

In the present study, we have evaluated the efficacy of heparin and a synthetic competitive thrombin inhibitor (2R,4R)-4-methyl-1-[N²-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-L-arginy1]-2-piperidinecarboxylic acid monohydrate (argatroban)14 in the prevention of arterial platelet-rich thrombus formation in a rabbit femoral artery eversion graft model. The results indicate that intravenous heparin is inefficient for the prevention of arterial occlusion. Arterial occlusion can, however, be persistently prevented with a short-term infusion of the synthetic thrombin inhibitor. These findings indicate that thrombin plays a pivotal role in platelet-mediated arterial occlusion and might suggest alternative approaches to the treatment of unstable atheromatous plaque.

Methods

Reagents

The synthetic thrombin inhibitor (2R,4R)-4-methyl-1-[N²-(3-methyl-1,2,3,4-tetrahydro-8-quinoline-
sulfonyl)l-arginyl]-2-piperidinecarboxylic acid monohydrate\textsuperscript{14} (Argatroban, Genentech, South San Francisco, California) was provided as a solution with a concentration of 0.5 mg active material per milliliter. Heparin was obtained from Elkins-Sinn Inc, Cherry Hill, New Jersey.

**Femoral Artery Eversion Graft Thrombosis Model**

New Zealand White rabbits, weighing 2.2–3.5 kg, were anesthetized with intravenous injection of sodium pentobarbital (35 mg/kg followed by 10-mg boluses when needed) by a marginal ear vein. After shaving both groins and right axilla, the right femoral artery and vein were exposed between the inguinal ligament and the distal bifurcation. The femoral vein was cannulated with a Silastic tubing (0.020 in. i.d., Dow Corning, Midland Michigan) for blood sampling. The right brachial artery was cannulated with a 22-gauge intracatheter (Deseret Medical Inc, Becton Dickinson, Sandy, Utah) for continuous blood pressure monitoring. A 4–6-mm segment of the right femoral artery was then excised, everted, and immersed in physiologic saline. The left femoral artery was then dissected, and the left superficial epigastric artery was cannulated with a Silastic tubing (0.012 in. I.D., Dow Corning) for intra-arterial administration of test material. The baseline blood flow in the left femoral artery was measured with an ultrasonic flowmeter (model T101, Transonic Sys., Inc., Ithaca, New York). The left femoral artery was transected midway between the inguinal ligament and the distal bifurcation, and the everted segment from the right femoral artery was inserted by end-to-end anastomosis using 12 interrupted sutures with 10-0 nylon (Sharpoint, Reading, Pennsylvania) under a surgical microscope (Wild M651, Heerbrugg, Switzerland) as previously described.\textsuperscript{15} After baseline blood sampling and 10 minutes before the release of the vessel clamps, the intravenous or intra-arterial infusion of heparin or argatroban was started. Intravenous heparin was given either as a bolus of 200 units/kg followed by 70 units/kg at hourly intervals (six rabbits) or as a bolus of 100 units/kg followed by infusion of 100 units/kg over 60 minutes (four rabbits) by the right femoral vein. Intravenous argatroban was given as a continuous infusion of 100 (seven rabbits) or 200 (six rabbits) \(\mu\)g/kg/min for 60 minutes. Intra-arterial heparin was infused at a rate of 200 units/kg over 60 minutes, and intra-arterial argatroban at a rate of 100 \(\mu\)g/kg/min for 60 minutes by the superficial epigastric artery, using a Harvard infusion pump (Syringe infusion pump 22, Harvard Apparatus, South Natick, Massachusetts). After release of the vessel clamps, the blood flow in the left femoral artery distal to the anastomosis was continuously recorded. When the flow decreased from a baseline value of 10–13 ml/min to less than 0.5 ml/min, the vessel was taken to be occluded. The primary end point of the study was the perfusion status measured 1 hour after the injection or the start of the infusion. If the everted arterial segment was open at the end of the 1-hour period, blood flow was monitored for an additional 1 hour in all animals and for up to 3 hours in a subgroup of animals. Blood samples were taken before the administration of drug, toward the end of the first hour after bolus injection or the start of the 1-hour infusion, and at hourly intervals during the additional follow-up period.

**Hematologic Assays**

Blood samples were obtained by the right femoral vein or right brachial artery. These samples were used for the determination of hematocrit, thrombin time, and partial thromboplastin time, using standard clinical chemistry assays. Platelet aggregation with 1 \(\mu\)l epinephrine (0.5 \(\mu\)M) and 14 \(\mu\)l arachidonic acid (0.5 \(\mu\)M) per 250 \(\mu\)l platelet-rich plasma was performed as described elsewhere.\textsuperscript{16}

Template bleeding times were performed on the shaved left foreleg using a spring-loaded blade device (Surgicutt International Technidyne, Edison, New Jersey). Incisions were made parallel to the antecebulal crease.

**Pathologic Examination**

At the end of the experiment, the everted segment and the adjacent femoral artery segments were excised and fixed overnight in 5\% formaldehyde. Intact excised arteries were processed for routine light microscopy. The arteries were sectioned longitudinally at multiple levels, and the slides were stained with hematoxylin and eosin. In nine rabbits with persistent patency, the everted segment was fixed in situ by perfusion with 80 mm Hg with 0.1 M cacodylate-buffered 0.25\% glutaraldehyde (pH 7.4). The everted arterial segment was excised, fixed for 24 hours, washed in buffer, dehydrated in graded alcohols, and critically point dried. The arterial segment was then divided longitudinally into two halves, mounted on an aluminum slab, sputter-coated with gold-palladium, and examined with scanning electron microscopy as previously described.\textsuperscript{17}

**Data Analysis**

Values are expressed as mean±SD. The significance of differences between groups was determined with Fisher’s exact test or Student’s \(t\) test.

**Results**

The effects of intravenous heparin and argatroban on femoral arterial eversion graft occlusion are summarized in Table 1. Intravenous administration of heparin did not prevent occlusion of the graft in 10 rabbits during an observation period of 1 hour, although it prolonged the thrombin time from 19±6 to more than 100 seconds and the partial thromboplastin time from 28±8 to more than 100 seconds (Table 2). Intravenous argatroban, however, prevented thrombosis in nine of 13 rabbits during the 1-hour infusion (0–60 minutes) (\(p=0.002\), vs. i.v. heparin), with persistent patency during the 1-hour follow-up period (60–120 minutes) in all nine rabbits.
and during the 3-hour follow-up period (120–240 minutes) in the three rabbits with prolonged monitoring. Two of seven rabbits receiving 100 μg/kg/min and two of six rabbits given 200 μg/kg/min occluded. Intra-arterial heparin infusion at a dose of 200 units/kg over 60 minutes prevented thrombosis of the graft in six of nine rabbits (p=0.003, vs. i.v. heparin) with persistent patency during the 1-hour follow-up in five of these six rabbits and during the 3-hour follow-up in the two rabbits with prolonged monitoring. Intra-arterial heparin infusion was associated with significant leaking from the graft sutures, which might explain the less pronounced prolongation of the thrombin and partial thromboplastin times in circulating blood as compared with i.v. heparin administration (Table 2).

Intra-arterial infusion of argatroban at a rate of 100 μg/kg/min for 60 minutes prevented thrombosis occlusion in all 10 animals during infusion (p=0.00001, vs. i.v. heparin) and during the follow-up periods (Table 1). Infusion of argatroban prolonged the thrombin time from 19±2 to more than 100 seconds toward the end of the infusion, which decreased to 75±29 after 1 hour and to 22±5 seconds after 3 hours (Table 2). Thus, patency was maintained after the end of the infusion, notwithstanding normalization of the thrombin time. Intra-arterial infusion of both heparin and argatroban produced a moderate decrease of both hematocrit and of systolic blood pressure of approximately 15% (data not shown).

Serial template bleeding times were performed in approximately 50% of the animals (Table 3). One hour after intravenous heparin infusion, the values of 4.0±0 minutes were not significantly different from baseline values of 4.1±0.5 minutes. Intravenous infusion of argatroban prolonged the bleeding time from 4.7±0.8 to 8.5±2.9 minutes (p=0.03) at the end of the 1-hour infusion period, which shortened to 5.8±3.6 minutes at the end of the 1-hour follow-up period and to 3.9±1.2 minutes at 3 hours. Intra-arterial heparin infusion did not influence the template bleeding time, whereas intra-arterial argatroban prolonged the bleeding time from 4.5±0.5 before to 6.8±1.0 minutes at the end of the infusion (p=0.06).

Results of femoral arterial blood flow measurements are summarized in Table 4. The baseline blood flow in the femoral artery in the experimental groups was 10–13 ml/min. After release of the vessel clamps, a significant hyperemic phase subsiding within 5 minutes was observed in all experimental groups. Toward the end of the first hour after release of the vessel clamps, the residual blood flow in the femoral artery grafts was significantly higher in the intra-arterial heparin group and in both argatroban groups than in the intravenous heparin group with consis-

### Table 1. Effects of Intravenous and Intra-arterial Heparin and Argatroban on Thrombotic Occlusion of the Femoral Arterial Eversion Graft

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Animals studied (n)</th>
<th>During infusion (0–60 min)</th>
<th>During 1-hr follow-up (60–120 min)</th>
<th>During additional 2-hr follow-up (120–240 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intradural infusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>10</td>
<td>10/10</td>
<td>. . .</td>
<td>. . .</td>
</tr>
<tr>
<td>Argatroban</td>
<td>13</td>
<td>4/13*</td>
<td>0/9</td>
<td>0/3</td>
</tr>
<tr>
<td>Intra-arterial infusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>9</td>
<td>3/9†</td>
<td>1/6</td>
<td>0/1</td>
</tr>
<tr>
<td>Argatroban</td>
<td>10</td>
<td>0/10‡</td>
<td>0/10</td>
<td>0/4</td>
</tr>
</tbody>
</table>

Data represent the number of animals with occluded grafts per total number studied in the group.

* p, 0.002, vs. i.v. heparin; †p, 0.003, vs. i.v. heparin; ‡p, 0.0001, vs. i.v. heparin; and p, 0.087, vs. intra-arterial heparin.

### Table 2. Effects of Heparin and Argatroban on Hemostasis Parameters

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Thrombin time (sec)</th>
<th>Partial thromboplastin time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>End of infusion</td>
</tr>
<tr>
<td>Intradural infusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>19±6</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Argatroban</td>
<td>16±3</td>
<td>94±12</td>
</tr>
<tr>
<td>Intra-arterial infusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>14±2</td>
<td>41±51</td>
</tr>
<tr>
<td>Argatroban</td>
<td>19±2</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Values are given as mean±SD.

*98±2 seconds in group receiving 200 units/kg bolus heparin followed by 70 units/kg bolus after 60 minutes (n=6) and 50±35 seconds in group receiving 100 units/kg bolus of heparin and an infusion of 100 units/kg over 60 minutes. For all other values in this table, no significant differences were observed in subgroups with different heparin or argatroban infusion schemes.

ND, not determined.
tently occluded segments. The arterial blood flow did not change significantly during the follow-up periods.

Light microscopy revealed that the occluding thrombus in the femoral arterial eversion grafts was rich in platelets and was intimately attached to the exposed adventitia (Figures 1A and 1B). In rabbits given intra-arterial heparin or argatroban and persistent patency of the grafted segment, light microscopy (Figures 2A and 2B) as well as scanning electron microscopy (Figures 3A and 3B) revealed a smooth luminal surface covered by layers of platelets without fibrin deposition.

**Discussion**

Coronary artery thrombosis at the site of a ruptured atheromatous plaque is a main common pathogenetic mechanism of ischemic coronary syndromes.\(^{18}\) Thrombus formation is initiated by platelet activation and aggregation at the site of plaque disruption.\(^{5,6}\) The mechanism of platelet activation is probably complex, comprising both initiation of platelet adhesion and aggregation as well as activation of the coagulation system by the exposed subendothelial structures.\(^{19}\) Both in animal models as well as in patients, however, antithrombotic therapy with aspirin, heparin, or the combination is not uniformly effective in the prevention of coronary thrombus formation.\(^{20,21}\) Two approaches have been used successfully to prevent reocclusion after thrombolytic therapy of coronary arterial thrombosis with recombinant tissue-type plasminogen activator (rt-PA), that is, maintenance infusion of rt-PA\(^{20}\) or the use of a potent antplatelet GPIIb/IIIa receptor antibody,\(^{10}\) but each approach can be associated with an increased bleeding risk. Indeed, prevention of reocclusion with rt-PA requires high doses and prolonged infusion, whereas the antibody is only efficient when given at a dose that saturates the receptor extensively.

In the present study, we have evaluated the efficacy of heparin and of a synthetic competitive thrombin inhibitor, argatroban, in the prevention of arterial platelet-rich thrombus formation in a rabbit femoral arterial eversion graft model, previously developed to study the effect of thrombolytic agents on platelet-rich thrombus.\(^{15}\) This everted graft represents a reproducible quantitative model of arterial thrombosis with material that is very rich in platelets. The thrombogenic stimuli produced by contact of the adventitial surface with the flowing blood are intense and presumably activate both the coagulation system (by exposed tissue factor\(^{22}\)) and blood platelets.\(^{19}\) Thrombus formation, however, seems to be insensitive to intravenous administration of large doses of heparin. Infusion of intravenous argatroban efficiently prevents platelet deposition in the femoral arterial graft, provided the infusion is given at a rate sufficient to induce a fourfold prolongation of the thrombin time in circulating blood. The superiority of argatroban over heparin is most conclusively demonstrated with intravenous administration of both agents. Indeed, with two variant schemes of minimal doses of heparin to consistently produce prolongation of the thrombin and partial thromboplastin times at 1 hour to more than 100 seconds, and with an argatroban infusion scheme that produced less extensive prolongation, the difference in frequency of occlusion was

**Table 3. Effects of Heparin and Argatroban on Template Bleeding Times**

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>n</th>
<th>Baseline</th>
<th>End of infusion</th>
<th>1-Hr follow-up</th>
<th>3-Hr follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intravenous infusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>4</td>
<td>4.1±0.5</td>
<td>4.0±0</td>
<td>4.0±0.9</td>
<td>3.5±0.8</td>
</tr>
<tr>
<td>Argatroban</td>
<td>5</td>
<td>4.7±0.8</td>
<td>8.5±2.9*</td>
<td>5.8±3.6</td>
<td>3.9±1.2</td>
</tr>
<tr>
<td>Intra-arterial infusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>3</td>
<td>3.7±0.3</td>
<td>3.3±0.3</td>
<td>3.5±0.5</td>
<td>ND</td>
</tr>
<tr>
<td>Argatroban</td>
<td>3</td>
<td>4.5±0.5</td>
<td>6.8±1.0†</td>
<td>6.2±2.3</td>
<td>3.3±1.8</td>
</tr>
</tbody>
</table>

Data represent mean±SD of bleeding times (min). *p, 0.03 vs. baseline; †p, 0.06, vs. baseline. ND, not determined.

**Table 4. Effects of Heparin and Argatroban on Femoral Arterial Blood Flow**

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Baseline (ml/min)</th>
<th>Hyperemic phase (% baseline)</th>
<th>End of infusion (% baseline)</th>
<th>1-Hr follow-up (% baseline)</th>
<th>3-Hr follow-up (% baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intravenous infusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>10±2.5</td>
<td>164±113</td>
<td>-3.0±4.2*</td>
<td>-2.0±2.9</td>
<td>1.3±2.2 (4)</td>
</tr>
<tr>
<td>Argatroban</td>
<td>12±5.5</td>
<td>180±54</td>
<td>38±39</td>
<td>37±34</td>
<td>43±43 (5)</td>
</tr>
<tr>
<td>Intra-arterial infusion</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>11±3.3</td>
<td>164±63</td>
<td>31±27</td>
<td>27±32</td>
<td>36±64 (3)†</td>
</tr>
<tr>
<td>Argatroban</td>
<td>13±4.2</td>
<td>205±86</td>
<td>48±17</td>
<td>38±20</td>
<td>88±117 (4)‡</td>
</tr>
</tbody>
</table>

Data represent mean±SD obtained in whole group, and in subgroup of studied animals at 3-hour follow-up as indicated in parentheses. *Values were expressed as percentage units of baseline flow relative to mechanical zero initially obtained by clamping of vessel; †two of three occluded; ‡includes one animal with a flow of 260%.
nevertheless highly significant ($p=0.002$). With intraarterial infusion of equivalent amounts over 1 hour, the difference in occlusion rates were less marked ($p=0.087$) but still in favor of argatroban (three of nine vs. none of 10 rabbits).

Our findings indicate that short-term infusion of argatroban results in a transient prolongation of the bleeding time, the thrombin time, and the partial thromboplastin time. Notwithstanding the short-lived in vivo effect of argatroban, however, consistent with its known plasma half-life of 5 minutes$^{23}$ and the partial normalization of the bleeding time within 1 hour and complete normalization within 3 hours, persistent patency of the everted femoral arterial grafts was observed throughout the experimental period. Pathologic examination of these grafts 1 hour or 3 hours after the end of the infusion revealed that the adventitial surface was covered by layers of platelets without platelet aggregation or fibrin deposition. Thus, although our study did not directly address the occurrence and mechanism of reduced thrombogenicity of the vessel wall, an apparent “passivation” of the thrombogenic surface seemed to occur rapidly and irreversibly. Our findings confirm and extend recent observations$^{12,13}$ suggesting that thrombin plays a pivotal role in the pathogenesis of platelet-rich arterial thrombosis. In aggregate, these studies indicate that thrombin is an important mediator in platelet-dependent arterial thrombus formation, irrespective of whether the thrombogenic stimulus is a foreign surface,$^{12}$ mural thrombus,$^{24}$ damaged vessel wall,$^{13}$ or adventitial tissue (this study). Moreover, all these studies have revealed that the platelet-mediated thrombus is relatively resistant to heparin. Three potential reasons for the higher efficacy of the synthetic inhibitors as compared with heparin have been proposed, including better local inactivation of thrombin bound to platelet receptors, more efficient inhibition of meizothrombin, which is resistant to the heparin antithrombin III complex, and faster diffusion of the low molecular weight synthetic inhibitors into the forming thrombus.$^{12}$

Although argatroban used in the present study and the synthetic chloromethyl ketone or hirudin used in the other studies prevent platelet-rich arterial throm-
bosis efficiently, their mechanism of thrombin inhibition is different. Indeed, whereas argatroban is a stable competitive inhibitor, the chloromethyl ketone and hirudin are irreversible inhibitors. Furthermore, although the pharmacokinetics and pharmacodynamics of these agents are probably significantly different, the short half-lives of these compounds (a few minutes duration)\textsuperscript{22,25} render their biological activity rapidly reversible.

The fact that vessel patency after argatroban infusion seemed to be maintained during the 1–3-hour follow-up period, during which the thrombin time had returned to baseline values, suggests that synthetic thrombin inhibitors might be useful adjunctive agents for the prevention of occlusion or reocclusion in association with angioplasty or thrombolytic therapy. Therefore, specific thrombin inhibitors constitute a potential alternative approach to antiplatelet agents for the treatment of unstable atheromatous plaque.

Acknowledgment

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References


KEY WORDS • thrombosis • platelets • argatroban
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