Time Course of the Effects of a Single Bolus Injection of F(ab')2 Fragments of the Antiplatelet GPIIb/IIIa Antibody 7E3 on Arterial Eversion Graft Occlusion, Platelet Aggregation, and Bleeding Time in Dogs

Robert Gabor Kiss, Hua Rong Lu, Tania Roskams, Ik-Kyung Jang, Edward F. Plow, Herman K. Gold, Désiré Collen

Abstract The time course of the effects of a single intravenous bolus injection of 10 mg/kg aspirin or 0.8 mg/kg F(ab')2 fragments of the monoclonal antiplatelet glycoprotein IIb/IIIa receptor antibody 7E3 [7E3-F(ab')2] on arterial occlusion, platelet aggregation, and bleeding time was studied in 30 dogs with an everted (inside out) carotid arterial segment inserted into the femoral artery. In the absence of an antiplatelet agent, the eversion grafts coagulated spontaneously with platelet-rich thrombus within 30 minutes. With aspirin, arterial occlusion persisted for 2 hours occurred in 5 of 10 dogs and cyclic occlusion and reflow in 4 animals; arterial occlusion was observed in all dogs at 24 hours. With 7E3-F(ab')2, arterial patency persisted throughout a 2-hour observation period in all of 10 dogs and for 24 hours in 4 of the 10 dogs. Contralateral eversion grafting 24 hours after aspirin or 7E3-F(ab')2 injection was associated with graft patency for 2 hours in 1 of 5 aspirin dogs and in 3 of 5 7E3-F(ab')2 dogs; patency persisted for 24 hours. In dogs grafted 48 hours after aspirin or 7E3-F(ab')2 injection, patency at 24 hours was seen in 0 of 5 dogs given aspirin and 3 of 5 dogs given 7E3-F(ab')2. The overall frequencies of arterial graft patency at 2, 24, 48, and 72 hours after study drug injection were significantly higher in the 7E3-F(ab')2 groups than in the aspirin groups (P<.005, n=10 in each group; P<.05, n=15; P<.005, n=15, and P=.05, n=5, respectively). ADP-induced ex vivo platelet aggregation was abolished after 7E3-F(ab')2 injection with partial recovery (approximately 20%; P=.01 versus baseline) within 24 hours and complete recovery (P=not significant versus baseline) within 48 hours. Pathological examination of patent everted grafts revealed significant residual mural thrombus in all groups. Thus, 7E3-F(ab')2 reduced platelet-rich arterial eversion graft thrombosis, and this effect persisted beyond normalization of bleeding time prolongation and inhibition of ex vivo platelet aggregation. Mural thrombus deposition still occurred with 7E3-F(ab')2, but vascular occlusion was markedly reduced. (Arterioscler Thromb. 1994;14:367-374.)

Key Words • arterial thrombosis • antiplatelet agents • anti-GPIIb/IIIa antibody • arterial patency

Platelet-mediated thrombosis constitutes a main pathogenetic mechanism of coronary artery occlusion and of reocclusion after successful thrombolytic therapy or angioplasty.1-7 Both antiplatelet agents3-13 and anticoagulants14-17 have been investigated in experimental animal models as a means of inhibiting platelet-mediated thrombus formation. The binding of fibrinogen to the platelet glycoprotein (GP) IIb/IIIa receptor constitutes a common pathway of human platelet aggregation by most physiological agonists.18,19 The inhibition of ligand binding to GP IIb/IIIa by means of specific monoclonal antibodies20 or by vipers venoms21-23 or synthetic peptides24-29 containing the Arg-Gly-Asp recognition sequence has indeed been shown to exert antithrombotic effects in vivo.30-34 The thrombogenicity of injured vessel walls and the required duration of antithrombotic treatment remain unknown, although it has been demonstrated that platelet deposition on injured vessel walls is a time-limited phenomenon.35-38

We have observed29 that a single bolus injection of 7E3-F(ab')2, fragments of the monoclonal antiplatelet GPIIb/IIIa antibody 7E3, 7E3-F(ab')2, not only enhances and accelerates arterial platelet-rich clot lysis with recombinant tissue-type plasminogen activator but also prevents both early (2-hour) and late (24-hour) reocclusion. More recently, a single intravenous bolus injection of 7E3-F(ab')2 was found to inhibit coronary artery occlusion after deep vessel wall injury for up to 6 days after its administration.40 In the present study we investigated the time course of the effects of a single intravenous bolus injection of 7E3-F(ab')2 on arterial occlusion, platelet aggregation, and bleeding time prolongation in an arterial eversion graft thrombosis model in the dog.

Methods

Reagents
7E3-F(ab')2, fragments of the murine monoclonal antibody 7E3 directed against the human platelet GPIIb/IIIa receptor
were from Centocor Inc. Heparin was from Novo, aspirin for intravenous use was from Synthelabo Benelux, and bovine thrombin (Topostasin) was from Hoffmann-La Roche.

**Everted Carotid/Femoral Arterial Thrombosis Model and Infusion Protocols**

The canine arterial eversion graft model used in the study has been previously described and is based on a rabbit femoral arterial eversion graft thrombosis model. Adult mongrel dogs weighing 12 to 20 kg were premedicated with 0.25 mg/kg fluanisone (Hynprom, Janssen Pharmaceutica) intramuscularly, anesthetized with 30 mg/kg intravenous sodium pentobarbital (Nembutal, CEVA) followed by 60-mg boluses when needed, intubated, and artificially ventilated. Cefazolin 1 g (Kezfol, Eli Lilly) was given intravenously. Catheters were inserted in the left jugular vein for blood sampling and in the brachial vein for infusion of study drugs. The right carotid artery was exposed and isolated, and a 3-cm segment was excised, everted, and immersed in physiological saline. The right femoral artery was then exposed in the inguinal region and any side branches were ligated. The baseline blood flow in the right femoral artery was measured with an electromagnetic (Medelad) or a Doppler (Transonic Systems Inc) flow probe. The everted segment from the right carotid artery was then inserted into the transected femoral artery by end-to-end anastomosis using a continuous suture with 7-0 nylon (DA Atraumatic, American Cyanamid Co, or Maxon, Cyanamid of Great Britain).

The study drugs were given either 10 minutes (groups Ia, Ib, IIA, and IIB) or 48 hours (groups Ic and IIC) before the release of the vessel clamps that occluded the proximal and distal ends of the transected femoral artery. Blood flow was restored in all 30 dogs to approximately 35% of baseline flow as adjusted by external constriction with a 2-mm-wide plastic constrictor applied 1 cm distal to the proximal anastomosis. Blood flow was continuously monitored with the flow probe for at least 2 hours and again at 24 hours, and when indicated at 48 hours. All animals received intravenous bolus injections of heparin (100 U/kg) 10 minutes before the release of the right femoral artery vessel clamps and a continuous infusion of 50 U/kg per hour for 2 hours.

The surgical procedure was performed under sterile conditions. During the 2-hour initial observation period, 500 mL 0.9% NaCl solution and 100 mL 5% glucose were infused. At the end of the experiment all catheters were removed, and the wounds were sutured. The dogs were given two separate subcutaneous injections of heparin (5000 U each) in the back. The trachea tubes were removed when spontaneous respiration had returned, and the animals were returned to their cages. All dogs were reinvestigated at 24 hours and at 48 hours, when indicated, to determine arterial graft patency. The carotid/femoral eversion graft segments were re-exposed, and the blood flow was determined with the flow probe. Twenty-four hours after study drug injection in groups Ia and IIa, the animals were subjected to carotid/femoral arterial eversion grafting on the left side with monitoring of the blood flow for 2 hours and again at 24 hours.

**Study Groups**

Thirty dogs were assigned to six study groups. Twenty dogs were given an intravenous bolus injection of 10 mg/kg aspirin (groups Ia-Ib+Ic) and 15 dogs received 0.8 mg/kg 7E3-F(ab')2 injection. The animal studies were conducted to conform to the guiding principles of the International Society for Thrombosis and Haemostasis.

**Ex Vivo Platelet Aggregation and Hemostasis Analyses**

Blood samples were collected in citrate (final concentration, 0.011 mol/L) before and 60 and 120 minutes after vessel clamp release. The results are expressed as mean±SEM. The significance of the differences between groups was compared by using the x² test for categorical data or Student’s t test for paired or unpaired values. A Kruskal-Wallis nonparametric ANOVA was performed on ranks of the ordered variable of arterial patency (0=persistent occlusion; 1=cyclical reflow and reocclusion after initial occlusion; 2=persistent patency after vessel clamp release). The correlations between the patency status (nonparametric variable) and bleeding time or platelet aggregation (parametric variables) were evaluated using Scheffé’s test.

**Results**

**Carotid/Femoral Arterial Graft Patency**

The results of right-side eversion graft blood flow measurements and patency status after the insertion of the everted carotid artery segment into the transected femoral artery are summarized in Table 1. Blood flow
was restored after the vessel clamp release to approximately 35% of baseline (from approximately 60 to 20 mL/min). Femoral arterial patency during the 2-hour initial observation period after vessel clamp release was categorized as persistent occlusion after initial occlusion, cyclic reflow and reocclusion after initial occlusion, or persistent patency after vessel clamp release. The time course of the right femoral artery eversion graft patency in the individual animals is schematically represented in Fig 1.

Intravenous bolus injection of 10 mg/kg aspirin in 10 dogs (groups 1a and 1b) was associated with persistent occlusion throughout the observation period in 5 dogs and with cyclic reflow and reocclusion in 4 animals; occlusion at 24 hours was observed in all of these animals. In 5 dogs that received the aspirin injection 48 hours before the grafting (group 1c), persistent occlusion during the initial observation period occurred in 4 dogs, and occlusion after 24 hours was observed in all 5 dogs. Intravenous bolus injection of 0.8 mg/kg 7E3-F(ab')2 in 10 dogs (groups 1a and 1b) was associated with persistent patency throughout the 2-hour initial observation period in all dogs, with persistent patency at 24 hours in 4 of these animals. In 5 dogs that received the 7E3-F(ab')2 injection 48 hours before the grafting (group 1c), cyclic occlusion and reflow during the initial 2-hour observation period occurred in 1 dog and persistent patency in the other 4 animals; 24 hours later, occlusion was observed in 2 of these 5 dogs.

The results of left-side eversion graft blood flow measurements and patency status are summarized in Table 1. Blood flow was restored after vessel clamp release to approximately 35% of baseline. The time course of the left carotid/femoral artery eversion graft patency in the individual animals is summarized in Fig 1. In the 5 dogs that underwent left-side grafting 24 hours after the vessel clamp release, occlusion was observed in 2 of these 5 dogs.
hours after aspirin injection (group Ia), persistent occlusion during the initial observation period was observed in 2 dogs, whereas the artery was occluded at 24 hours in 4 of the 5 dogs. In the 5 dogs subjected to left carotid/femoral arterial eversion grafting 24 hours after injection of 7E3-F(ab')2 (group Ia), persistent patency during the initial observation period was observed in 3 dogs, and a patent artery was observed in 3 of the 5 dogs at 24 hours.

Several significant differences in carotid/femoral arterial eversion graft patency were obtained in these groups. The mean femoral arterial blood flow in the groups given 7E3-F(ab')2 (groups Ha and lib) was similar to that in the dogs given 10 mg/kg aspirin at 1 hour after clamp release (groups Ia and Iib). The platelet count did not differ significantly between 7E3-F(ab')2 and aspirin. Patency status during the initial 2-hour observation period occurred more frequently with aspirin (18 of 20) than with 7E3-F(ab')2 (3 of 20) (P<.0004 by χ² analysis). A Kruskal-Wallis analysis, with patency status at 2, 24, 48, and 72 hours after study drug injection as described in “Methods,” yielded P<.0005, n=10 in each group; P<.05, n=15; P<.005, n=15; and P=.05, n=5, respectively, for patency differences between 7E3-F(ab')2 and aspirin. Patency status during the initial 2-hour observation period and at 24 hours was not significantly different (P=.16 and P=.48, respectively) between the groups given 7E3-F(ab')2 10 minutes before vessel clamp release (groups Ia and Iib) or 48 hours before grafting (group Iic).

No significant correlation was found between eversion graft patency and ex vivo platelet aggregation or bleeding time.

### Table 3: Plasma Fibrinogen Levels and Activated Partial Thromboplastin Times

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline 2 H</th>
<th>24 H</th>
<th>48 H</th>
<th>72 H</th>
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<td>Ia</td>
<td>120±10</td>
<td>180±30</td>
<td>350±110</td>
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<tr>
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<td>85±10</td>
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<td>Iib</td>
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<td>260±50</td>
<td>400±70</td>
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<tr>
<td>Ilc</td>
<td>110±10</td>
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<td>260±60</td>
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<table>
<thead>
<tr>
<th>Group</th>
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<th>24 H</th>
<th>25 H</th>
<th>48 H</th>
<th>72 H</th>
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<td>40±17</td>
<td>17±1</td>
<td>42±11</td>
<td>17±1</td>
</tr>
<tr>
<td>Ib</td>
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<td>26±8</td>
<td>36±11</td>
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<td>17±1</td>
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<td>17±0</td>
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<tr>
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<td>39±9</td>
<td>19±2</td>
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<td>15±2</td>
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<td>68±22</td>
<td>17±1</td>
<td>32±9</td>
<td>23±3</td>
</tr>
</tbody>
</table>

Data represent the mean±SEM of five experiments.

### Ex Vivo Platelet Aggregation and Hemostasis Analyses

ADP-induced ex vivo platelet aggregation was slightly but significantly reduced in the aspirin-treated animals (groups Ia+Ib+Ic). In contrast, complete inhibition of platelet aggregation was observed in all animals given 7E3-F(ab')2, with a partial recovery within 24 hours and nearly complete recovery (P=not significant versus baseline) at 48 hours. Platelet aggregation induced with epinephrine and arachidonic acid was markedly and significantly suppressed both in dogs given aspirin (groups Ia+Ib+Ic) and in dogs given 7E3-F(ab')2 (groups Ia+Ib+Ic). The platelet count did not change markedly in any of the groups (Table 2).

Fibrinogen levels increased significantly in all groups in response to the surgical challenge (Table 3). The activated partial thromboplastin time was 1.5 to 4 times prolonged during the initial 2-hour observation period but was normal at 24 hours despite subcutaneous heparin administration (Table 3).

### Template Bleeding Times

Template bleeding times (Table 4) measured before injection of study drugs averaged 1.0 to 2.0 minutes. Bleeding times at 60 minutes were prolonged slightly but significantly after intravenous aspirin injection (P<.05 versus control). Administration of 7E3-F(ab')2 resulted in a maximal prolongation of the bleeding time at 60 minutes that normalized within 24 to 48 hours.

### Pathology

The results of light microscopic analysis are summarized in Table 5. In all dogs given aspirin, occlusive...
thrombus was observed that consisted of platelet-rich or mixed platelet-rich and erythrocyte-rich thrombus. In dogs given 7E3-F(ab')2, extensive residual mural thrombus consisting of platelet-rich or mixed platelet- and erythrocyte-rich material was found. In no instance were fully patent arteries without mural thrombus observed. Typical examples of everted segments are represented in Fig 2.

Discussion

Coronary artery thrombosis at the site of a ruptured atheromatous plaque is a main common pathogenetic mechanism of ischemic coronary syndromes. Thrombus formation is initiated by platelet activation and aggregation at the site of plaque rupture. 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. 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. Furthermore, although platelet deposition on injured vessel walls is a time-limited phenomenon, the time course of vessel wall thrombogenicity remains largely unknown.

In the present study, we compared the time course of the effects on platelet-mediated thrombosis, ex vivo platelet aggregation, and bleeding time prolongation of a single bolus injection of 7E3-F(ab')2 fragments of the platelet GPIIb/IIIa receptor blocking monoclonal antibody 7E3 in a platelet-mediated arterial and aorta graft thrombosis model in the dog. This antibody, at the dose used, saturates at least 90% of the platelet GPIIb/IIIa receptors and exerts potent antithrombotic effects on platelet-mediated arterial thrombosis in vivo.

The arterial eversion graft model in the dog is a simplified variant of the rabbit eversion femoral artery graft model. The 3-cm carotid eversion graft inserted into a transected femoral artery is highly thrombogenic and reproducible. Stable thrombosis, primarily created by the deposition of platelet-rich material, occurs spontaneously within 17±16 minutes (mean±SD) notwithstanding full heparinization (100 U/kg per hour) to attain a three- to fivefold prolongation of the activated partial thromboplastin time.

Bolus injection of 0.8 mg/kg 7E3-F(ab')2 efficiently prevented or delayed platelet deposition in the carotid/femoral arterial eversion graft. Indeed, although blood flow after vessel clamp release was similar in the aspirin and 7E3-F(ab')2 groups, it was significantly higher at all time points thereafter in the 7E3-F(ab')2 groups. The

<table>
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<th>1 H</th>
<th>2 H</th>
<th>24 H</th>
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<th>26 H</th>
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<td>1.3±0.3</td>
<td>3.7±0.5*</td>
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<td>5.6±2.9</td>
<td>2.4±0.6</td>
<td>2.2±0.4</td>
</tr>
</tbody>
</table>

Data represent the mean±SEM of five experiments.

*P<.05 vs control.
patency status was also significantly better in the antibody group than in the aspirin group.

As expected, injection of 0.8 mg/kg 7E3-F(ab')2 prolonged the bleeding time to more than 30 minutes and abolished ADP-induced platelet aggregation. The prolongation of the bleeding time and the inhibition of platelet aggregation had partially recovered at 24 hours and completely at 48 hours, but this was not associated with reocclusion. Aspirin in combination with heparin resulted in a modest but significant prolongation of the bleeding time.

Histological examination 24 and 48 hours after grafting and up to 72 hours after study drug administration revealed extensive mural thrombus in both groups, but to a lesser extent in the 7E3-F(ab')2 group. These observations agree with our previous findings.39 In the present study, we could not determine whether these thrombi were formed early during the course of the procedure and were maintained or were formed later, possibly in association with the recovery of platelet function. Mural thrombus formed in the 7E3-F(ab')2-treated animal might contribute to the apparent passivation of the thrombogenic character of the everted vessel that sustains the patency of this vessel over a prolonged time. Thus, the present results suggested that thrombogenicity is relatively transient. In the accompanying article47 the kinetics and mechanisms of the passivation process are considered further.

In summary, our results confirmed and extended the observation that a single bolus injection of 7E3-F(ab')2 prevents reocclusion not only during an initial 2-hour observation period but also after 24 and 48 hours, when the prolongation of the bleeding time and the inhibition of platelet aggregation are largely or completely normalized.

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