Contribution of Platelets and the Vessel Wall to the Antithrombotic Effects of a Single Bolus Injection of Fab Fragments of the Antiplatelet GPIIb/IIIa Antibody 7E3 in a Canine Arterial Eversion Graft Preparation

Robert Gabor Kiss, Jean Marie Stassen, Hans Deckmyn, Tania Roskams, Herman K. Gold, Edward F. Plow, Désiré Collen

Abstract The contribution of platelets and the vessel wall to the antithrombotic effects of a single intravenous bolus injection of 0.8 mg/kg Fab fragments of the monoclonal antiplatelet glycoprotein IIb/IIIa receptor antibody 7E3 (7E3-Fab), combined with continuous heparin anticoagulation (100 U/kg bolus and 50 U/kg per hour), was studied in a canine preparation consisting of an everted (inside out) carotid arterial segment that had been inserted into a transected femoral artery. In all 6 control dogs without antibody, persistent or transient eversion graft occlusion occurred during an initial 2-hour observation period, and 5 of the 6 grafts were occluded at 24 hours. In 6 dogs given 7E3-Fab 24 hours before receiving an everted carotid artery segment from a donor dog, cyclic occlusion and reflow occurred in all dogs, whereas the grafts were patent at the end of a 2-hour observation period in 5 of the 6 dogs (P = .056 versus control). When transfused back to the donor dogs, the patent eversion segments showed brief periods of cyclic occlusion and reflow within 2 hours in 3 of 5 dogs (P = .034 versus control), whereas all of the 5 eversion segments were patent at 24 hours (P < .005 versus control). When platelet concentrates (prepared 48 hours after injection of the antibody fragment from approximately 50% of the blood volume of the dogs given 7E3-Fab) were transfused into animals with eversion grafts 10 minutes before vessel clamp release, persistent or transient occlusion was observed within 2 hours in all 5 dogs (P = not significant versus control), but 4 of these 5 grafts were patent at 24 hours (P = .045 versus control). ADP-induced platelet aggregation and bleeding times remained normal throughout the eversion grafting experiments in all dogs. Thus, long-term or even short-term exposure of eversion grafts to platelets treated with 7E3-Fab conferred reduced thrombogenicity. (Arterioscler Thromb. 1994;14:375-380.)

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

Platelet-mediated thrombosis is a main cause of arterial occlusion after atherosclerotic plaque rupture.1,2 Patency can be restored by thrombolytic therapy or angioplasty combined with conjunctive anticoagulant and/or antiplatelet agents.3 The temporal dependency of the thrombogenicity of the damaged vessel wall has been demonstrated in several studies,4-6 but the mechanisms of "passivation" remain largely unknown. We have observed7 that a single bolus injection of 0.8 mg/kg F(ab')2 fragments of the monoclonal antiplatelet glycoprotein (GP) IIb/IIIa receptor antibody 7E3, 7E3-F(ab'),8 abolished early platelet-mediated occlusion of arterial eversion grafts in a canine preparation. Persistent graft patency was observed in approximately 50% of the animals for up to 72 hours after study drug injection, a time at which both the template bleeding times and platelet aggregation had returned to baseline levels.7 However, persistent graft patency was associated with moderate to extensive mural thrombosis. Similar results were also obtained in an electrolytic injury model in dogs in which 7E3 produced sustained coronary artery patency over a 6-day period.9

In the present study we addressed two specific questions. First, does exposure of the everted graft to blood pretreated 24 hours earlier with the antibody fragment reduce its thrombogenicity? Second, do platelets pretreated 48 hours earlier with the antibody fragment protect against graft occlusion? The results suggested that briefly exposed everted segments acquire a reduced thrombogenicity and that pretreated platelets protect against thrombosis. Although our study did not elucidate mechanisms, it demonstrated that the thrombogenicity and the hemostatic function of platelets are dissociable and that the passivating effects of pharmacological agents will be an important consideration in preventing rethrombosis.

Methods

Fab fragments of the murine monoclonal antibody 7E3 raised against the human platelet GPIIb/IIIa receptor but cross-reacting with the canine receptor 7E3-Fab8 were from Centocor Inc. 7E3-Fab fragments were preferred over 7E3-
F(ab’); because of their more rapid clearance from the blood and because the Fab fragment has been selected for further clinical development. Heparin was from S.A. Novo Nordisk Pharma and thrombin from Sigma Chemical Co.

Everted Carotid/Femoral Arterial Graft Thrombosis

The model was used essentially as described elsewhere, but with the following modifications. A central venous catheter was placed and tunneled to the upper part of the back for connection to a portable elastometric infusion pump (Hemapump, Block Medical Inc.). The everted right carotid artery segment was inserted into the transected femoral artery by end-to-end anastomosis using 0.5-cm-long polyethylene tube connectors with a 2-mm internal diameter, and no external constrictor was applied. After releasing the vessel clamps that occluded the proximal and distal ends of the transected femoral artery, the blood flow was restored to 50% to 80% of baseline flow as revealed by a Doppler flow probe (Transonic Systems Inc.). Blood flow was monitored continuously for the initial 2 hours and again at 24 hours. All animals received a bolus injection 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 with the portable infusion pump until the end of the experiment. The surgical procedure was performed under sterile conditions. During the surgery and the 2-hour initial observation period, 300 mL 0.9% NaCl solution was infused. Surgical wounds were closed with skin staples (Proximate-II TW35, Ethicon). The tracheal tube was removed when spontaneous respiration had returned, and the animals were returned to their cages. All dogs were reinvestigated with the flow probe at 24 hours to determine arterial graft patency.

Study Groups and Drug Allocation

Twenty-two dogs were assigned to four study groups. Six control dogs with an everted carotid/femoral arterial eversion graft received only a continuous infusion of heparin, started 10 minutes before removal of the vessel clamps, without exposure to antiplatelet agents.

Six dogs were given a single 0.8 mg/kg intravenous bolus injection of the 7E3-Fab antibody fragment without heparin. Twenty-four hours after the injection, these dogs received an everted carotid artery segment from a donor dog that was monitored in the antibody-treated dog with the flow probe for 2 hours during heparin anticoagulation. Five of these six grafts were patent at the end of the 2-hour observation period and were transferred back to the donor dogs, where they were monitored with the flow probe for 2 hours and again after 24 hours during continuous heparin anticoagulation. All observations in this “graft donor” group were made after the transplantation of the graft back into these animals.

Five of the dogs given 7E3-Fab were bled 48 hours after the antibody injection, and their blood was collected into an acid-citrate-dextrose (ACD) solution (15 g/L citric acid, 25 g/L trisodium citrate, and 20 g/L glucose in sterile distilled water; blood/ACD ratio, 9:1) for isolation of blood platelets. The amount of blood collected represented approximately 6% of the body weight. The blood was centrifuged at 100g for 10 minutes at room temperature; platelet-rich plasma was decanted and centrifuged at 850g for 15 minutes, and the platelet pellet was carefully resuspended in 25 mL of a mixture of saline and ACD (9:1, vol/vol). An estimated 56% of the dogs’ circulating platelets (range, 40% to 85% relative to the platelet number originally present in the circulating blood) was recovered. The platelet concentrate of each dog was transfused to a single recipient dog with a carotid/femoral eversion graft 10 minutes before removal of the vessel clamps. This group is referred to as the “platelet recipient” group. Graft patency was monitored with the flow probe for 2 hours and again at 24 hours. The animal studies were conducted to conform to the guiding principles of the International Society of Thrombosis and Haemostasis.

Ex Vivo Platelet Aggregation

Blood samples were collected in citrate (final concentration, 0.011 mol/L) before and 60 and 120 minutes after vessel clamp release and again at 24 hours (and at 48 hours in 7E3-Fab–treated dogs). These samples were used for measurements of platelet count and ex vivo platelet aggregation induced with threshold final concentrations of ADP (9.8±0.05 μmol/L, n=22) or of the combination of epinephrine (1 μmol/L) and arachidonic acid (0.5 mmol/L). Platelet counting was performed with an automatic cell counter (Cell-Dyn 610, Sequoia Turner). Platelet aggregation was performed within 1 hour after blood sampling by using a standard aggregometer (Elvi 840). Plasma was stored frozen for subsequent determination of fibrinogen and activated partial thromboplastin time (aPTT).

Template Bleeding Times

Template bleeding times were measured before and at 60 and 120 minutes after vessel clamp release and again at 24 hours (and at 48 hours in 7E3-Fab–treated dogs). The bleeding time was measured by using a standardized spring-loaded device (Simplate-II, General Diagnostics) applied to the volar surface of the foreleg. The region of the incision was washed, shaved, and dried before performance of the first bleeding time.

Pathology

At the end of the experiments the dogs were killed with an overdose of pentobarbital. The evasion graft segments were removed, fixed overnight in 10% neutral formaldehyde, and embedded in paraffin. The sections were sectioned transversely, stained with hematoxylin and eosin, and evaluated microscopically. The extent of thrombosis and the composition of the thrombus were graded as described elsewhere.

Statistical Analyses

The results are expressed as mean±SEM. The significance of the differences between groups was compared by using 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=occlusive occlusion; 1=cyclical reflow and reocclusion after initial occlusion; and 2=persistent patency after vessel clamp release) and on ranks of the ordered variable of the extent of thrombosis (0=occlusive thrombus; 1=partial thrombus; and 2=mural thrombus or patent artery).

Results

Carotid/Femoral Arterial Eversion Graft Patency

The results of the arterial blood flow measurements and carotid/femoral arterial eversion graft patency are summarized in Table 1. Blood flow was restored after vessel clamp release to 50% to 90% of baseline (from 51±6 to 33±5 mL/min). Femoral artery patency during the observation period was categorized as persistent occlusion after initial occlusion, cyclical reflow and reocclusion after initial occlusion, or persistent patency. The time course of the evasion graft patency in the individual animals is schematically represented in Fig 1.

In the 6 control dogs given continuous heparin infusion but no antiplatelet agents, persistent or transient occlusion of the evasion graft occurred during the 2-hour initial observation period, and 5 of the 6 grafts were occluded at 24 hours. Six dogs given 7E3-Fab received an everted carotid artery segment from a donor dog 24 hours after antibody injection. These grafts were then monitored with the flow probe for 2 hours. During this observation period, all of the trans-
planted grafts underwent transient occlusion (P = .056 versus control by Kruskal-Wallis analysis), and 5 of the 6 grafts were patent at the end of the observation period. The 5 patent eversion grafts were then transferred back to the original donors and monitored for patency during concomitant heparin anticoagulation. This resulted in brief periods of cyclic occlusion and reflow within 2 hours in 3 of the 5 dogs (P = .034 versus controls) and persistent patency in the other 2 animals. Patency of all 5 eversion grafts was observed at 24 hours (P < .005 versus controls). Blood flow during the initial 2-hour observation period was significantly higher in the 7E3-Fab–treated group (P = .02 versus control at 1 hour) and in the eversion graft donor group (P < .001 versus control at 1 hour) than in the control group.

Platelet concentrates were prepared from approximately 50% of the circulating blood of the dogs given 7E3-Fab 48 hours after the injection of the antibody fragment. These platelet preparations were then transfused into dogs with carotid/femoral arterial eversion grafts 10 minutes before removal of the vessel clamps. This resulted in persistent or transient occlusion within a 2-hour initial observation period in all of 5 dogs (P = not significant versus control). Thus, during the initial observation period, these animals were very similar to the control group in terms of patency status. This similarity was also reflected in the blood flow parameters of the recipient animals (Table 1). However, in strong contrast to the control group, in which 5 of the 6 grafts were occluded at 24 hours, 4 of the 5 grafts in the recipient group were patent at 24 hours (P = .045 versus control). In three control experiments with platelets from dogs not given 7E3-Fab, persistent eversion graft occlusion occurred during the initial 2-hour observation period, and all grafts were occluded at 24 hours (data not shown).

### Ex Vivo Platelet Aggregation and Blood Analyses

Ex vivo ADP-induced platelet aggregation (Table 2) was nearly completely abolished in dogs given 7E3-Fab 2 hours after antibody injection, but it had fully recovered at 24 and 48 hours. Arachidonic acid– and epinephrine-induced platelet aggregation was only slightly altered at 2 hours in the 7E3-Fab group, with a complete recovery at 24 and 48 hours (data not shown). Platelet aggregation was also somewhat reduced in the control group and in the graft donor groups, possibly as a result of the intense heparin anticoagulation. The platelet count did not change markedly during the experimental period.

The fibrinogen levels and aPTT values are summarized in Table 3. Intense anticoagulation with heparin induced an at least threefold prolongation of the aPTT throughout the experiments.

### Template Bleeding Times

Administration of 7E3-Fab resulted in a significant prolongation of the template bleeding time 2 hours after the injection, with complete recovery at 24 hours (Table 4). In the other groups the bleeding times were in the normal range throughout the experiments.

### Pathology

Fig 2 illustrates a typical light-microscopic view of an occluded (top) and a patent reperfused (bottom) vessel. The results of the microscopic evaluation of carotid/femoral eversion grafts with extent and composition of the thrombus graded as defined elsewhere are summarized in Table 5. The extent of arterial thrombosis was significantly different between the control group and the graft donor group (P = .002 by Kruskal-Wallis analysis) and between the control group and the platelet recipient groups (P = .009 by Kruskal-Wallis analysis).
several instances patent arteries with minor localized mural thrombus were observed (Fig 2, bottom).

### Discussion

Numerous and large clinical studies (eg, TIMI-1, ECSCG-1, GISSI-1, ISIS-2, and GUSTO) have been performed over the past few years to evaluate and optimize thrombolytic therapy. Reocclusion after successful coronary artery recanalization has been a significant clinical problem with all available thrombolytic regimens. With tissue plasminogen activator as a thrombolytic agent, it has been estimated that occlusive rethrombosis occurs in 5% to 25% of patients. Investigations in animal models have suggested that thrombus formation after recanalization with thrombolytic agents are platelet rich (for references, see Reference 20). Accordingly, manipulation of platelet function, either by directly interfering with the capacity of the platelets to aggregate or by inhibiting the generation of platelet agonists, such as the thrombus itself, represents a rational approach toward reducing rethrombosis.

Several recent studies suggest that the monoclonal antibody 7E3, when directed toward the GPIIb/IIIa receptor, can significantly suppress the occurrence of rethrombosis. Gold et al have shown that 7E3 sustains patency for a prolonged time (6 days) in a canine electrolytic model of thrombosis. We have shown that 7E3 induces long-term patency and prevents rethrombosis in a platelet-rich thrombosis model. The present study further documented the capacity of 7E3 to prevent reocclusion over a prolonged time and extended these observations in several ways. First, the graft transplantation experiments suggested that a relatively short-term exposure of the thrombogenic stimulus (the everted graft) in a 7E3-treated dog has a significant influence on the occurrence of occlusion. Although the thrombogenic character of the everted graft was not fully abrogated by the 2-hour exposure in the 7E3-treated animal (cyclic reocclusion and reflow were observed in 3 of 5 animals after reininsertion of the everted graft into donor animals), patency was consistently observed at 24 hours. Thus, a 2-hour exposure to 7E3-treated platelets may be sufficient to initiate, if not fully achieve, a passivation of the everted segment. Second, the passivating effect of 7E3 could be transferred with the platelets from donor to recipient animals. Since free 7E3-F(ab) fragments are removed during platelet isolation and the platelets with bound 7E3 circulate normally, it is reasonable to assume that 7E3 may prevent occlusion in the recipient animals. Taken together, these first two observations suggest the exciting possibility that short-term exposure of platelets in vitro to 7E3 (or other GPIIb/IIIa antagonists), followed by reinfusion, could reduce the tendency to reocclusion.

A third point suggested by the present study is that the passivating effect of 7E3 is achieved without prolongation of bleeding time or alteration of platelet aggregation in response to ADP. Thus, the passivating effects of 7E3 in the graft donor group and in the platelet recipient group were achieved without compromising the hemostatic function of the platelets. This observation indicated that the thrombotic function of platelets can be dissociated from their hemostatic function and further suggested that the hemostatically active platelet may, in fact, be important in preventing reocclusion of the everted grafts. Thrombus was present in the grafts taken from the donor dogs 24 hours after the antibody injection; thus, rather than being a thrombogenic stimulus, a thrombus containing platelets with bound 7E3 may contribute to the passivation phenomenon.

There are several potential explanations for the normal hemostatic function of platelets with bound 7E3. First, platelets contain an intracellular pool of GPIIb/
Ilia that is expressed on the cell surface in association with platelet secretion. This newly expressed GPIIb/IIIa is functionally active.\(^{23,24}\) Second, a sufficient number of newly formed platelets, unexposed to 7E3, may enter the circulation over 24 to 48 hours to support the hemostatic functions of the platelets. Third, there may be some displacement of 7E3 from the surface of platelets, sufficient to allow recovery of hemostatic function, without full recovery of thrombogenic potential. Whatever the mechanism, the dissociation of the hemostatic from the thrombogenic properties of platelets could be valuable from a therapeutic standpoint.

### TABLE 4. Template Bleeding Times

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Baseline</th>
<th>2 H</th>
<th>24 H</th>
<th>26 H</th>
<th>48 H</th>
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<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>2.5±0.4</td>
<td>3.5±0.82</td>
<td>2.3±0.2</td>
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<td>...</td>
</tr>
<tr>
<td>7E3-Fab treated</td>
<td>6</td>
<td>2.4±0.3</td>
<td>7.1±1.4*</td>
<td>3.5±0.8†</td>
<td>4.0±0.5</td>
<td>2.1±0.2</td>
</tr>
<tr>
<td>Graft donor</td>
<td>5</td>
<td>1.5±0.1</td>
<td>2.6±0.5</td>
<td>2.5±0.4</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Platelet recipient</td>
<td>5</td>
<td>1.4±0.2</td>
<td>2.6±0.3</td>
<td>2.8±0.3</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM.

*P<.05 vs baseline.
†P=.36 vs baseline.

![Fig 2. Light microscopy photomicrographs showing transverse sections of everted carotid femoral artery segments. Top, An occluded vessel with a mixed platelet-rich and erythrocyte-rich thrombus; bottom, a reperfused vessel with a minor platelet-rich mural thrombus (original magnification ×50).](image-url)
### Table 5. Results of Light Microscopic Analysis of Everted Carotid/Femoral Arterial Eversion Grafts

<table>
<thead>
<tr>
<th>Group</th>
<th>Sequence Number</th>
<th>Patency Status</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control dogs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>OT</td>
<td>ER</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>OT</td>
<td>ER</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>OT</td>
<td>ER</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>OT</td>
<td>PR</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>OT</td>
<td>MPE</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>OT</td>
<td>MPE/WBC</td>
<td></td>
</tr>
<tr>
<td>Graft donor dogs</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>PA/MT</td>
<td>MPE</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>PT</td>
<td>MPE</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PA/MT</td>
<td>MPE</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>PA/MT</td>
<td>MPE</td>
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</tr>
<tr>
<td>5</td>
<td>PA/MT</td>
<td>MPE</td>
<td></td>
</tr>
<tr>
<td>Platelet recipient dogs</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>PA/MT</td>
<td>MPE</td>
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</tr>
<tr>
<td>5</td>
<td>OT</td>
<td>MPE</td>
<td></td>
</tr>
</tbody>
</table>

OT indicates occlusive thrombus; ER, erythrocyte rich; PR, platelet rich; MPE, mixed with interfaced platelet-rich and erythrocyte-rich zones; WBC, white blood cells; PA, patent artery; MT, mural thrombus; and PT, partially occlusive thrombus.

The results of the present study, taken together with those of the accompanying study, indicate that concomitant heparin anticoagulation potentiates the antithrombotic effect of 7E3-Fab fragments but that heparin anticoagulation alone can prevent neither early nor delayed eversion graft occlusion. Indeed, in the control group of the present study, which received intense continuous anticoagulation but no antibody, graft occlusion was consistently observed; in the antibody group of the accompanying study, which received subcutaneous heparin without aPTT prolongation at 24 hours, extensive occlusive or subocclusive thrombus was observed. In contrast, in the combined antibody and intravenous heparin groups (graft donor and platelet recipient groups) of the present study, the extent of mural thrombus at 24 hours was markedly reduced.

In summary, the present study suggested that a single intravenous bolus injection of 7E3-Fab in conjunction with continuous therapeutic heparin anticoagulation prevents early and delayed arterial eversion graft thrombosis by mechanisms in which early arterial wall passivation and reduced thrombogenicity of platelets exposed to the antibody play a significant role.

### Acknowledgments

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### References

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