Effects of Abciximab, Ticlopidine, and Combined Abciximab/Ticlopidine Therapy on Platelet and Leukocyte Function in Patients Undergoing Coronary Angioplasty

Becky J. Fredrickson, PhD; Nancy A. Turner, BS; Neal S. Kleiman, MD; Nikki Graziadei, BS; Kelly Maresh, RN; Mary Ann Mascelli, PhD; Mark B. Effron, MD; Larry V. McIntire, PhD

Background—Abciximab and ticlopidine reduce adverse cardiovascular events after percutaneous transluminal coronary angioplasty (PTCA). The goal of the current study was to determine if combined abciximab/ticlopidine therapy inhibits arterial thrombosis more effectively than either treatment alone. The effect of each therapy on platelet-leukocyte interactions was also investigated.

Methods and Results—Whole blood samples from 14 patients undergoing PTCA who received abciximab therapy, ticlopidine therapy, or both treatments were evaluated using dynamic experimental systems. Mural thrombus formation under arterial shear conditions (1500 s^{-1}) was determined in a parallel plate flow chamber. Shear-induced platelet aggregation was evaluated using a cone-and-plate viscometer at a shear rate of 3000 s^{-1}. Of the 3 treatments, combined abciximab/ticlopidine therapy produced the most consistent reduction in shear-induced platelet aggregation and the most prolonged inhibition of mural thrombosis. Three days after PTCA, abciximab/ticlopidine treatment decreased mural thrombus formation to \( \approx50\% \) of baseline values. Abciximab treatment alone inhibited mural thrombosis for only 1 day after PTCA, whereas ticlopidine treatment alone had no significant effect. Two hours after PTCA, abciximab therapy significantly decreased the number of circulating platelet-neutrophil aggregates but significantly enhanced P-selectin–mediated leukocyte adhesion on the collagen/von Willebrand factor–platelet surface.

Conclusions—Combined therapy with abciximab and ticlopidine has a prolonged inhibitory effect on mural thrombosis formation relative to either treatment alone. Further, we demonstrated an unexpected effect of abciximab in enhancing P-selectin–mediated leukocyte adhesion. (Circulation. 2000;101:1122-1129.)

Key Words: platelets | leukocytes | thrombosis | abciximab | ticlopidine | angioplasty

Pathological arterial thrombosis limits the success of percutaneous transluminal coronary angioplasty (PTCA) by causing short-term ischemic episodes and contributing to vessel restenosis. The molecular mechanisms that support pathological thrombosis depend on the local fluid mechanical environment in the vessel. Under arterial shear conditions, platelet adhesion to the exposed subendothelium of vessels injured by PTCA is supported by the integrin glycoprotein (GP) IIb-IIIa receptor. Subsequent platelet aggregation on the vessel wall is mediated by soluble von Willebrand factor (vWF) bridging GP IIb-IIIa receptors on platelets. Under shear conditions characteristic of stenotic vessels, platelet aggregates also form as a direct result of shear-induced platelet activation. This high shear–induced platelet aggregation depends on vWF binding to GP IIb-IIIa receptors. Although platelets are the primary mediators of arterial thrombosis, leukocytes are activated by PTCA and contribute to the vascular response. To reduce the clinical complications of PTCA, a number of antithrombotic agents have been developed. One of the most successful compounds is abciximab, a murine/human chimeric monoclonal antibody fragment directed against the human platelet GP IIb-IIIa (\( \alpha_{IIb}\beta_{3} \)) receptor. Abciximab also binds to the integrin \( \alpha_{V}\beta_{3} \) and Mac-1 (\( \alpha_{M}\beta_{2} \)) receptors. The phase III Evaluation of IIb-IIIa platelet receptor antagonist 7E3 in Preventing Ischemic Complications (EPIC) study demonstrated that abciximab decreased acute ischemic events after PTCA by 35% and that it decreased restenosis 6 months after PTCA by 23%. Ticlopidine has also been successful in reducing adverse cardiac events after PTCA with stent implantation. Although the effects of abciximab on platelet aggregation occur within a matter of minutes, the effects of ticlopidine are not seen until several days after administration. Therefore, the simultaneous administration of these drugs may be beneficial. The objective of the current study was to compare...
combined abciximab and ticlopidine therapy with either treatment alone by monitoring platelet and leukocyte function in patient whole blood samples before PTCA and up to 14 days after PTCA. This was done using dynamic experimental systems that model 2 distinct molecular mechanisms that lead to pathological arterial thrombosis. Shear-induced platelet aggregation (SIPA) was evaluated using patient whole blood in a cone-and-plate viscometer. Mural thrombus formation from patient whole blood was detected in a parallel plate flow chamber. Combined abciximab/ticlopidine therapy provided the most extended and consistent reduction of thrombosis. In the presence of abciximab, we observed an unexpected interaction between leukocytes and platelets in mural thrombosis.

Methods

Compounds
Abciximab (c7E3, Reo-Pro, Centocor, Inc/Eli Lilly & Co) is a murine/human chimeric monoclonal antibody fragment directed against the human platelet GP IIb-IIIa receptor.14,15 Ticlopidine (Ticlid, Syntex) is an orally active agent that inhibits ADP-induced platelet aggregation.20 The antiplatelet effects of ticlopidine become maximal 5 to 7 days after long-term administration and continue 4 to 8 days after the cessation of therapy.

HuEP5C7.g4 was prepared by Dr Ellen Berg of Protein Design Labs, Inc, Mountain View, Calif. HuEP5C7.g4 is a humanized monoclonal antibody (IgG1) that binds both P- and E-selectin.23 PSL-275 is a monoclonal antibody (IgG1) directed against P-selectin glycoprotein ligand-1 (PSGL-1, CD162).24 Both the whole PSL-275 antibody and the Fab fragment were used. S12 binds P-selectin but does not block function.25 These compounds were added to normal donor blood for 10 minutes immediately before parallel plate flow chamber and flow cytometry experiments in the following concentrations: 20 μg/mL HuEP5C7.g4, 10 μg/mL PSL-275, 20 μg/mL S12, and 5 μg/mL abciximab.

Patients
A total of 14 patients undergoing high-risk PTCA who were scheduled to receive abciximab, ticlopidine, or both treatments were enrolled in the study. Patients included both men and women aged 40 to 72. None of the patients had received prior abciximab treatment, and no patient had received ticlopidine within the 2 months before PTCA. Additionally, no patient had a known bleeding or platelet disorder in the 6 months before PTCA, and none of the patients had a known hyperactivity to murine proteins or an evolving myocardial infarction. Patients were selected for each group according to the anatomical propriety of the target coronary lesion for stent placement. The study was approved by the Institutional Review Board for Human Subject Research for Baylor College of Medicine and Affiliated Hospitals, and all patients signed informed consent documents.

Medical Treatment
Of the 14 patients enrolled in the study, 5 received abciximab therapy, 5 received ticlopidine therapy, and 4 received both treatments. Standard abciximab therapy includes a 0.25 mg/kg bolus injection immediately before PTCA, which is followed by a 12-hour, 10 μg/min infusion. Standard ticlopidine therapy begins with a 500-mg initial dose 12 to 36 hours before PTCA, which is followed by 250 mg BID for 30 days and includes stent placement during PTCA. All patients who received ticlopidine or abciximab/ticlopidine also received a JJIS stent. Patients who received abciximab...
alone did not receive a stent. All patients received 325 mg of aspirin daily beginning ≥24 hours before PTCA.

Blood Collection
Patient whole blood samples were collected at 7 time points. Collection points from before PTCA included a baseline sample 12 to 36 hours before PTCA and a sample <1 hour before PTCA. Other samples were collected 2 hours, 24 hours, 3 days, 7 days, and 14 days after treatment. The sample taken <1 hour before PTCA was obtained from the femoral arterial sheath. At all other time points, patient samples were obtained by venipuncture. All samples were anticoagulated with sodium citrate (0.38% wt/vol). Patient hematocrits were monitored at each time point, and they remained nearly constant throughout the study. The fluorescent dye mepacrine (quinacrine dihydrochloride, Sigma) was added to the blood used in parallel plate experiments at a concentration of 10 μmol/L.

SIPA
SIPA was evaluated using whole blood and a cone-and-plate viscometer (Ferranti-Shirley 781, Ferranti Electric, Inc). Whole blood samples were sheared for 30 s at a shear rate of 3000 s⁻¹ (shear stress=140 dynes/cm²) at room temperature. Aliquots of pre- and post-shear samples measuring 5 μL were taken and immediately fixed with 5 μL of 2% formaldehyde in Dulbecco’s PBS (Sigma). The fixed samples were combined with saturating concentrations of CD62-PE (anti–P-selectin, Becton Dickinson Immunocytometry Systems) and CD42a-FITC. Background fluorescence was determined using saturating concentrations of IgG-PE and CD42a-FITC. To measure the number of circulating platelet-leukocyte aggregates, 5 μL of patient or normal donor whole blood was incubated for 20 minutes with saturating concentrations of CD42a-FITC and CD45-PerCP (leukocyte anti–HLA-1, Becton Dickinson Immunocytometry Systems). Background fluorescence was determined using saturating concentrations of CD45-PerCP but no CD42a-FITC. Fixed samples were analyzed using the FACScan flow cytometer. The number of platelet-leukocyte aggregates was determined by identifying the leukocytes by PerCP-fluorescence, gating on each leukocyte subpopulation,²⁹,³⁰ and measuring the amount of FITC-fluorescence above background in each leukocyte subpopulation (Figure 1).

Flow Cytometric Studies
P-selectin expression on circulating platelets and the number of circulating platelet-leukocyte aggregates were quantified using flow cytometry, as previously described.²⁶–²⁸ To determine P-selectin expression on platelets, 5 μL of patient or normal donor whole blood was incubated with saturating concentrations of CD62-PE (anti–P-selectin, Becton Dickinson Immunocytometry Systems) and CD42a-FITC. Background fluorescence was measured using saturating concentrations of IgG-PE and CD42a-FITC. To measure the number of circulating platelet-leukocyte aggregates, 5 μL of patient or normal donor whole blood was incubated for 20 minutes with saturating concentrations of CD42a-FITC and CD45-PerCP (leukocyte anti–HLA-1, Becton Dickinson Immunocytometry Systems). Background fluorescence was determined using saturating concentrations of CD45-PerCP but no CD42a-FITC. Fixed samples were analyzed using the FACScan flow cytometer. The number of platelet-leukocyte aggregates was determined by identifying the leukocytes by PerCP-fluorescence, gating on each leukocyte subpopulation,²⁹,³⁰ and measuring the amount of FITC-fluorescence above background in each leukocyte subpopulation (Figure 1).

Parallel Plate Flow Chamber and Digital Image Processing
Mural thrombus formation was determined using a parallel plate flow chamber and epifluorescent video microscopy, as previously described.²³–²⁴ Glass coverslips were coated with 200 μL of a suspension of type I acid-insoluble collagen (1100 μg/mL), which was prepared from a bovine Achilles’ tendon (Sigma).³¹ Whole blood was drawn through the chamber for 1 minute at a constant flow rate, producing a wall shear rate of 1500 s⁻¹. Mural leukocyte-platelet interactions were determined by counting the total number of leukocytes rolling on the collagen/vWF–platelet surface in a single field of view over the 1-minute flow period. Leukocytes that contacted the surface but were immediately carried
back to the center of the flow stream without even transiently rolling were not included in the numbers reported.

Statistics
Results are reported as mean ± SEM. The significance of the difference between means was determined by ANOVA using Fisher’s protected least significant difference test.

Results
SIPA
SIPA was significantly reduced 2 hours after PTCA with abciximab/ticlopidine (A), abciximab (B), or ticlopidine (C) treatment. Results are reported as percentage of neutrophils forming neutrophil-platelet aggregates in patient whole blood samples. Values are mean ± SEM, n = 3 to 5. Relative difference between means is significant (P < 0.05) between *baseline and time point, § abciximab/ticlopidine and ticlopidine, and ¥ abciximab and ticlopidine.

Leukocyte-Platelet Aggregates in Normal Donor Blood, With and Without Abciximab

<table>
<thead>
<tr>
<th>Leukocyte Subpopulation</th>
<th>Leukocyte-Platelet Aggregates, %</th>
<th>Control</th>
<th>With Abciximab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil</td>
<td>5.4 ± 0.7</td>
<td>8.9 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>Monocyte</td>
<td>21.9 ± 2.0</td>
<td>27.7 ± 5.0</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>2.9 ± 0.5</td>
<td>2.8 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

*Percentages refer to the number of leukocytes bound to platelets relative to the total number of leukocytes in the sample.

Figure 5. Inhibition of circulating neutrophil-platelet aggregates by abciximab/ticlopidine (A), abciximab (B), or ticlopidine (C) treatment. Mural thrombus formation was evaluated after a 60-s perfusion of patient whole blood at a wall shear rate of 1500 s⁻¹ over a collagen coverslip. Values are mean ± SEM, n = 3 to 5. P < 0.05, * Difference between means is statistically significant (P < 0.05) between baseline and time point.

Figure 6. Inhibition of circulating neutrophil-platelet aggregates by abciximab/ticlopidine (A), abciximab (B), or ticlopidine (C) treatment. Mural thrombus formation was evaluated after a 60-s perfusion of patient whole blood at a wall shear rate of 1500 s⁻¹ over a collagen coverslip. Values are mean ± SEM, n = 3 to 5. P < 0.05, * Difference between means is statistically significant (P < 0.05) between baseline and time point.

Platelet Activation
Abciximab/ticlopidine treatment significantly decreased the activation of circulating platelets relative to baseline 2 hours, 7 days, and 14 days after PTCA (Figure 4A). At these time points, the percentage of circulating, activated platelets was reduced to 50% of baseline values. Abciximab or ticlopidine treatment alone had no effect on circulating platelet activation at any time point (Figure 4, B and C).

Leukocyte-Platelet Aggregates
The percentage of circulating neutrophil-platelet aggregates was decreased by > 70% relative to baseline values 2 hours after PTCA with abciximab/ticlopidine or abciximab treatment alone (Figure 5, A and B). Compared with ticlopidine treatment alone, abciximab and abciximab/ticlopidine treatment resulted in significantly fewer circulating neutrophil-
platelet aggregates at this time point (Figure 5). In patients receiving abciximab, the percentage of circulating neutrophil-platelet aggregates was significantly greater relative to baseline in the samples taken immediately before PTCA and 14 days after PTCA. Monocyte-platelet and lymphocyte-platelet aggregates were also measured. The percentage of monocyte-platelet aggregates increased by 44% relative to baseline 7 days and 14 days after PTCA in samples from patients receiving ticlopidine treatment alone. The addition of abciximab to whole blood drawn from normal donors did not significantly change the percentage of leukocyte-platelet aggregates measured by flow cytometry (Table). However, relative to patient whole blood samples, the percentage of circulating leukocyte-platelet aggregates was much lower in the control samples from normal donors.

Mural Thrombus Formation
Mural thrombus formation was significantly decreased relative to baseline patient samples 3 days after PTCA with abciximab/ticlopidine treatment and 1 day after PTCA with abciximab treatment alone (Figure 6, A and B). One day after PTCA, treatment with both abciximab/ticlopidine and abciximab alone decreased the total number of platelets deposited by 84% and 58%, respectively, relative to baseline samples. In contrast, ticlopidine treatment alone had no effect (Figure 6C). Three days after PTCA, abciximab/ticlopidine treatment effectively reduced the total number of platelets deposited to 45% of baseline values, whereas either treatment alone had no effect (Figures 6 and 7). The total area of the collagen surface covered by platelets was also significantly reduced 3 days after PTCA with abciximab/ticlopidine treatment (Figure 8A); at this time point, the total surface area covered by platelets was 50% that of baseline coverage. Abciximab treatment alone significantly reduced the total surface coverage by platelets to 66% of baseline values 2 hours after PTCA (Figure 8B). Ticlopidine treatment alone did not significantly reduce the total surface area covered by platelets at any time point (Figure 8C).

P-Selectin/PSGL-1–Mediated Leukocyte Rolling
During parallel plate experiments, high numbers of leukocytes rolling on the collagen surface were observed with abciximab/ticlopidine or abciximab treatment 2 hours after PTCA (Figures 9 and 10). No significance differences between the numbers of rolling leukocytes were observed between treatment with abciximab/ticlopidine or abciximab alone. Rolling leukocytes were not observed immediately after the initiation of flow; they were seen after a monolayer
of platelets had been deposited on the collagen coverslip. Firm adhesion of leukocytes was not observed. When ticlopidine was administered alone, rolling leukocytes were not observed in significant numbers; instead, rather large thrombi developed on the collagen/vWF surface (Figures 9 and 10).

To confirm the specificity of leukocyte rolling on the collagen/vWF–platelet surface, flow chamber experiments using normal donor blood were performed in the presence of abciximab and monoclonal antibodies against P-selectin and PSGL-1. As observed in patient samples, leukocytes from normal donors rolled in significant numbers on the collagen/vWF–platelet surface in the presence of abciximab. When HuEP5C7.g4 or PSL-275 was added to the whole blood samples containing abciximab, leukocyte rolling was reduced by >80% (Figure 11). When HuEP5C7.g4, PSL-275, or S12 was added to whole blood without abciximab, leukocyte rolling was not significant (data not shown).

**Discussion**

Of the 3 treatments, combined abciximab/ticlopidine therapy produced the most consistent reduction in SIPA (Figures 2 and 3). The effect of abciximab/ticlopidine was most pronounced 2 hours after PTCA, when SIPA was reduced by 50% relative to baseline samples. At this time point, patients were still receiving a 10 μg/min abciximab infusion, and abciximab treatment alone produced a similar reduction in SIPA. Combined abciximab/ticlopidine therapy also produced the most prolonged reduction in mural thrombus formation (Figures 6 and 8). Three days after PTCA, abciximab/ticlopidine treatment decreased mural thrombus formation to ~50% of baseline values. Abciximab treatment alone effectively inhibited mural thrombosis for only 1 day after PTCA, whereas ticlopidine treatment alone did not decrease mural thrombus formation significantly at any time point studied. These results suggest that the sustained clinical benefit of abciximab treatment alone may be a direct effect of inhibiting mural thrombus growth during the critical period before vessel wall passivation, thereby reducing the local concentration of platelet-derived products, including ADP, thrombin, and platelet-derived growth factor, which stimulate the neointimal hyperplasia. In most patients, the vessel wall becomes passivated in ~2 days. However, in some patients, passivation may not occur for up to 8 days. Therefore, the prolonged reduction in mural thrombus formation produced by combined abciximab/ticlopidine treatment may further decrease clinical events after PTCA relative to abciximab treatment alone.

Two hours after PTCA, treatment with abciximab/ticlopidine or abciximab alone resulted in significant numbers of leukocytes rolling on the collagen surface (Figures 9 and 10). The high number of leukocytes rolling on the platelet monolayer 2 hours after PTCA could be a result of the increased availability of free leukocytes circulating in the blood stream, because both abciximab/ticlopidine and abciximab treatment alone decreased the number of leukocyte-platelet aggregates in the circulation (Figure 5). This is unlikely, however, because the addition of abciximab to normal donor blood did not decrease the number of leukocyte-platelet aggregates but did promote leukocyte rolling on the platelet monolayer (Figure 11). A more likely hypothesis is that the platelet monolayer forms using the GP Ib-IX-V complex and creates a reactive carpet of P-selectin that enhances the recruitment of leukocytes to the surface by binding leukocyte PSGL-1. In support of this, we showed that leukocyte rolling is almost completely inhibited by anti-P-selectin or PSGL-1 monoclonal antibodies (Figure 11).
Adhesion of leukocytes to sites of arterial injury after PTCA may aggravate the endothelial damage through the release of proteolytic enzymes and other toxic substances. Additionally, leukocyte secretory products may potentiate platelet activation and amplify the thrombotic process. The effects of increased P-selectin–mediated leukocyte adhesion may be offset by the cross-reactivity of abciximab with the integrin Mac-1. Mac-1 supports monocyte and neutrophil adhesion to intercellular adhesion molecule-1 and fibrinogen, which are both present in the subendothelial matrix. Abciximab significantly reduces Mac-1–mediated binding to both of these ligands. Therefore, on a denuded vessel, abciximab may enhance P-selectin–mediated adhesion while diminishing adhesion supported by intercellular adhesion molecule-1 and fibrinogen.

In summary, we demonstrated that combined abciximab/ticlopidine therapy significantly inhibits mural thrombus formation, as assessed in a parallel plate flow chamber, for 3 days after PTCA. This is a significant increase over the effective duration of either treatment alone. The antiplatelet effects of the combined therapy are still evident 7 and 14 days after PTCA, as indicated by the reduction in the percentage of circulating, activated platelets relative to baseline values. Additionally, we found that abciximab treatment decreases the percentage of circulating leukocyte-platelet aggregates but promotes leukocyte-platelet interactions on a collagen/vWf–platelet surface.

Acknowledgments
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References

Figure 10. Images of platelet deposition and leukocyte rolling on collagen/vWf–platelet surface 2 hours after PTCA. Leukocytes (●) and (▲) were only observed to roll after formation of platelet monolayer, which is designated by → in A. Platelet monolayers formed when whole blood samples from patients receiving either abciximab/ticlopidine treatment (A) or abciximab treatment alone (B) were perfused. Ticlopidine (C) treatment resulted in platelet thrombi with essentially no leukocyte involvement. Experimental conditions were identical to those in Figure 6.

In summary, we demonstrated that combined abciximab/ticlopidine therapy significantly inhibits mural thrombus formation, as assessed in a parallel plate flow chamber, for 3 days after PTCA. This is a significant increase over the effective duration of either treatment alone. The antiplatelet effects of the combined therapy are still evident 7 and 14 days after PTCA, as indicated by the reduction in the percentage of circulating, activated platelets relative to baseline values. Additionally, we found that abciximab treatment decreases the percentage of circulating leukocyte-platelet aggregates but promotes leukocyte-platelet interactions on a collagen/vWf–platelet surface.

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