Dissociation of Glycoprotein IIb/IIIa Antagonists From Platelets Does Not Result in Fibrinogen Binding or Platelet Aggregation

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Background—The primary mechanism of action of glycoprotein (GP) IIb/IIIa antagonists is inhibition of the final common pathway of platelet aggregation: fibrinogen binding to the GP IIb/IIIa complex. However, it has been reported that induction of fibrinogen binding and platelet aggregation is an intrinsic prothrombotic property of low-dose GP IIb/IIIa antagonists. These apparently paradoxical results have been extensively referenced in the cardiology literature.

Methods and Results—By platelet aggregation and flow cytometry, we demonstrate that (1) dissociation of GP IIb/IIIa antagonists (abciximab, tirofiban, epitifibatide, or xemilofiban) from platelets does not result in platelet aggregation; (2) tirofiban and epitifibatide can induce a fibrinogen-binding–competent conformation of the GP IIb/IIIa receptor, but stable fibrinogen binding does not occur without fixation; (3) the slow off-rate of abciximab exposes only a small proportion of unblocked GP IIb/IIIa receptors at any time, and these also fail to stably bind fibrinogen; and (4) the GP IIb/IIIa antagonist–induced fibrinogen binding in some previously reported experiments was probably the result of artifactual thrombin generation.

Conclusions—Under physiological conditions, GP IIb/IIIa antagonists currently in clinical use do not have an intrinsic activating property that results in platelet aggregation or stable fibrinogen binding to GP IIb/IIIa. (Circulation. 2001;104:1374-1379.)

Key Words: platelets ■ inhibitors ■ glycoproteins ■ fibrinogen ■ receptors

Glycoprotein (GP) IIb/IIIa antagonists are clinically proven antithrombotic agents in the settings of coronary artery disease and percutaneous coronary interventions.1 The primary mechanism of action of GP IIb/IIIa antagonists is inhibition of the final common pathway of platelet aggregation: fibrinogen or von Willebrand factor binding to the GP IIb/IIIa complex.1 However, Peter et al2 recently reported that induction of fibrinogen binding and platelet aggregation are intrinsic prothrombotic properties of low-dose GP IIb/IIIa antagonists. The marked clinical benefit of the intravenous GP IIb/IIIa antagonists (abciximab, epitifibatide, tirofiban) in patients with acute coronary syndromes and in those patients undergoing percutaneous coronary interventions suggests that GP IIb/IIIa antagonist–induced platelet activation is not a clinically relevant issue in these settings. However, the apparently paradoxical results of Peter et al2 have been extensively referenced in the cardiology literature1,3–12 as a potential causative factor in (1) the higher mortality rates in patients receiving low-dose oral GP IIb/IIIa antagonists in 3 different multicenter trials (Evaluation of Oral Xemilofiban in Controlling Thrombotic Events [EXCITE], Orbofiban in Patients with Unstable Coronary Syndromes [OPUS], and Sibrafiban Versus Aspirin to Yield Maximum Protection from Ischemic Heart Events Post-Acute Coronary Syndromes [SYMPHONY]);1,3–5,7,9,11,12 (2) the recently reported disappointing results of abciximab in the GUSTO-IV trial of unstable angina,1 and (3) abciximab-induced pseudothrombocytopenia.10 Therefore, in the present study, we reexamined the findings of Peter et al2 by using a number of independent methods.

Methods

Blood Collection

Blood was collected from consenting, aspirin-free, healthy donors into 3.2% citrate Vacutainer tubes (Becton Dickinson). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by centrifuging blood at 100g or 800g, respectively, for 15 minutes at 22°C.

Platelet Aggregation

Aggregation of PRP was analyzed in a Chrono-Log 560Ca Lumi-Aggregometer. For control aggregations, PRP was equilibrated (37°C, 5 minutes), and aggregation was initiated with 20 μmol/L ADP (Sigma-Aldrich). According to the method of Peter et al,3 PRP was preincubated (22°C, 30 minutes) with either buffer or GP IIb/IIIa antagonist: abciximab (Centocor), tirofiban (Merck), epitifibatide (Eli Lilly), or xemilofiban (Pfizer).
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Fibrinogen binding was assessed with the use of FITC-conjugated F26 (gift of Dr Harvey Gralnick), a monoclonal antibody that detects a receptor-induced binding site on fibrinogen bound to GP IIb/IIIa. Citrated PRP was diluted 1:10 in PPP. Diluted PRP (10 μL) was incubated with 5 μL abciximab, epifibatide, or tirofiban and 5 μL containing FITC-F26 (11.7 μg/mL) and, as a platelet identifier, peridinin chlorophyll protein (PerCP)-labeled CD42a (anti-GPIIb, Becton Dickinson) (1:20) (all concentrations final). Both the GP IIb/IIIa antagonist solutions and the antibody mixture were prepared in modified HEPES-Tyrode’s buffer, pH 7.4, with 2 mmol/L CaCl2 and 2 mmol/L MgCl2. After 1 hour at 22°C, samples were fixed with 1% formaldehyde and analyzed in a FACScalibur flow cytometer (Becton Dickinson). Platelets were identified by their characteristic forward and side light scatter profile and staining with PerCP-CD42a. A positive analysis region was set on flow cytometric histograms depicting F26 binding to unstimulated platelets in the presence of 10 mmol/L EDTA, so that 1% of the platelets were in the positive range. In some experiments, fibrinogen binding to platelets was assessed by use of the method of Peter et al, with the following modifications. PerCP-CD42a was added to citrated whole blood as a platelet identifier. Whole blood was then diluted 1:50 in modified Tyrode’s buffer, pH 7.4, with 2 mmol/L CaCl2 and 2 mmol/L MgCl2. After 1 hour at 22°C, samples were fixed with 1% formaldehyde and analyzed in a FACScalibur flow cytometer (Becton Dickinson). Platelets were identified by their characteristic forward and side light scatter profile and staining with PerCP-CD42a. A positive analysis region was set on flow cytometric histograms depicting F26 binding to unstimulated platelets in the presence of 10 mmol/L EDTA, so that 1% of the platelets were in the positive range.

In other experiments, FITC-conjugated fibrinogen was used to directly assess GP IIb/IIIa antagonist–induced platelet activation. Whole blood diluted 1:20 with 10 mmol/L HEPES Tyrode’s buffer, pH 7.4, mixed with FITC-conjugated fibrinogen (100 μg/mL), PerCP-CD42a (as a platelet identifier), and phycoerythrin-conjugated P-selectin–specific monoclonal antibody 1E3 (Dako), was incubated (22°C, 30 minutes) with either abciximab (6 μL) and FITC-labeled polyclonal chicken antifibrinogen antibody (BioPool International) (4 μL). Samples were diluted in 1% formaldehyde in 10 mmol/L PBS, pH 7.4, or 10 mmol/L HEPES-buffered saline, pH 7.4.

In other experiments, FITC-conjugated fibrinogen was used to determine the proportion of available (unoccupied) receptors and their capacity to bind fibrinogen, aliquots taken before and after addition of abciximab were incubated (22°C, 10 minutes) with excess FITC-conjugated abciximab (40 μg/mL) or FITC-conjugated monoclonal antibody F26 (8 μg/mL) with or without 20 μmol/L ADP/20 μmol/L thrombin receptor activating peptide (TRAP, Calbiochem), then fixed with 1% formaldehyde in PBS. Non-specific F26 binding was measured in parallel in the presence of 10 mmol/L EDTA. Dot plots of Fl-2 (RPE-CD41) versus Fl-3 (PerCP-CD42a) were used to establish nonoverlapping gates for the RPE-CD41–labeled control platelets and PerCP-CD42a–labeled abciximab-treated platelets.

Fibrinogen Binding to Antagonist-Treated, Fixed Platelets

The ability of abciximab (10 μg/mL), epifibatide (1 μg/mL), or tirofiban (50 ng/mL) to induce a fibrinogen binding–competent conformation of GP IIb/IIIa was tested by fixing platelets in the presence of these antagonists and assessing fibrinogen binding after washing to remove the antagonist. Platelets in PRP were fixed and washed as described by Peter et al and bound fibrinogen was assessed by flow cytometry with FITC-F26, with PerCP-CD61 as the platelet identifier. An alternate method (Du et al20) was used to fix washed platelets in the presence of antagonists. Binding of fibrinogen or PAC1 (a monoclonal antibody mimic of fibrinogen’s activation-dependent binding to platelets) to these antagonist-treated, fixed, washed platelets was determined by incubating cells (22°C, 30 minutes) with FITC-fibrinogen (75 μg/mL) or FITC-PAC1 (Becton Dickinson). Non-specific binding was determined by addition of excess epifibatide.

Results

Platelet Aggregation

The ability of low-dose GP IIb/IIIa antagonists to act as partial agonists stimulating platelet aggregation was investigated. No aggregation was observed when PRP was preincubated with low-dose abciximab (0.1 μg/mL), 2 mmol/L CaCl2/2 mmol/L MgCl2, and the sample stirred in an aggregometer (Figure 1). Varying abciximab concentration (from 0.05 to 0.3 μg/mL) or preincubation time (15 minutes to 2 hours) similarly did not result in platelet aggregation (data not shown). Preincubation of PRP with comparable low doses of tirofiban (0.5 ng/mL), epifibatide (0.01 μg/mL), or the active metabolite of xemilofiban, SC-54701 (1 nmol/L).
Flow Cytometric Evaluation of GP IIb/IIIa Antagonist–Induced Fibrinogen Binding to Unfixed Platelets

Flow cytometry was used as a more sensitive method to assess the putative intrinsic activating property of GP IIb/IIIa antagonists. Diluted PRP was incubated with abciximab (0.001 to 10 μg/mL), tirofiban (0.005 to 50 ng/mL), or eptifibatide (0.0001 to 1 μg/mL) for 1 hour in the presence of the anti–receptor-induced binding site monoclonal antibody F26 (to detect GP IIb/IIIa–bound fibrinogen). The percentage of F26-positive platelets was 1.60.1% (mean ± SD, n = 3) in the presence of buffer alone (without EDTA) and was not significantly increased at any concentration of GP IIb/IIIa antagonist, whereas ADP (20 μmol/L) produced 91.5% F26-positive platelets (Figure 2).

Whole blood diluted in buffer (45 minutes, 22°C), then incubated with a polyclonal antifibrinogen antibody and increasing concentrations of abciximab as described, showed a biphasic pattern of fibrinogen binding, reproducing the results of Peter et al2 (Figure 3, solid bars). However, omission of calcium from (data not shown) or addition of hirudin to the dilution buffer eliminated fibrinogen binding at all concentrations of abciximab (Figure 3, open bars). No fibrinogen binding was observed at any abciximab concentration when the blood was used immediately after dilution, even when the diluting buffer contained calcium (data not shown).

Finally, FITC-fibrinogen (rather than FITC-F26) and a P-selectin–specific monoclonal antibody (an independent marker of platelet activation) were used to evaluate platelet activation by low-dose GP IIb/IIIa antagonists. Low concentrations of the GP IIb/IIIa antagonists (sufficient to partially block ADP-induced fibrinogen binding) did not induce fibrinogen binding or P-selectin expression above control levels (Table).

Modeling In Vivo Abciximab Dissociation

Taken together, the above data indicate that the binding and dissociation of abciximab, tirofiban, eptifibatide, and SC-54701 over a period of 30 to 60 minutes at 22°C across a wide range of antagonist concentrations does not lead to activated GP IIb/IIIa and stable fibrinogen binding. Considering the rapid on- and off-rates of tirofiban but not abciximab, these conditions would result in a large proportion of GP IIb/IIIa that previously had antagonist bound and then dissociated, whereas only a very small percentage of receptors would have had abciximab dissociated. We therefore designed an experiment to maximize and quantify the proportion of receptors to which abciximab had bound and dissociated. In addition, these experiments were carried out at 37°C in plasma, thereby more closely approximating in vivo conditions. Platelet GP IIb/IIIa was blocked by incubation with abciximab (1 μg/mL) for 30 minutes at 37°C, then
diluted 100-fold in PPP and abciximab allowed to dissociate over 6.5 hours at 37°C. Binding of FITC-abciximab revealed that only \( \approx 10\% \) of the surface exposed GP IIb/IIIa was unoccupied (ie, \( \approx 90\% \) blocked) after 30 minutes of incubation with 1 \( \mu g/mL \) abciximab (Figure 4). Dilution of these blocked platelets in plasma and continued incubation at 37°C increased the percentage of available receptors to 75% at 420 minutes (Figure 4). Likewise, ADP/TRAP-stimulated fibrinogen binding to cells treated with abciximab for 30 minutes was reduced to 6% of control (94% inhibited), and dilution resulted in a slow, progressive recovery of fibrinogen binding, increasing to 57% of control at 420 minutes (43% inhibited) (Figure 4). Most importantly, despite this demonstrable dissociation of abciximab from receptor, no antagonist-induced fibrinogen binding was observed in the absence of ADP/TRAP (Figure 4).

**Fibrinogen Binding to GP IIb/IIIa Antagonist–Treated, Fixed, Washed Platelets**

Because potential receptor-activating properties of GP IIb/IIIa antagonists could be masked by antagonist blockade of the receptor, we assessed platelet surface fibrinogen binding and activation of GP IIb/IIIa on fixed antagonist-treated platelets that were subsequently washed to remove antagonist. Platelets fixed in the presence of tirofiban or eptifibatide, then washed according to the method of Peter et al \(^2\) in buffer containing fibrinogen and calcium, showed increased binding of the antifibrinogen antibody F26 (Figure 5A) but were clumped and difficult to resuspend. In contrast, platelets fixed in the presence of abciximab and washed by the same procedure suspended readily after centrifugation and showed no F26 binding (Figure 5A).

Similar results were obtained when washed platelets were fixed in the presence of abciximab, eptifibatide, or tirofiban according to the method of Du et al \(^15\) except that the absence of fibrinogen prevented aggregate formation during washing and improved platelet recovery. By this method, eptifibatide-fixed and tirofiban-fixed platelets showed increased binding of FITC-labeled fibrinogen compared with control platelets, whereas abciximab-fixed platelets showed no fibrinogen binding (Figure 5B).

**Discussion**

In this study, we demonstrate by several independent methods that (1) dissociation from platelets of abciximab, tirofiban, eptifibatide, or the active metabolite of xemilofiban does not result in platelet aggregation, fibrinogen binding, or surface GP IIb/IIIa Antagonists Do Not Induce Fibrinogen Binding or P-Selectin Expression

<table>
<thead>
<tr>
<th>Fibrinogen</th>
<th>P-Selectin</th>
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<tbody>
<tr>
<td>Unstimulated</td>
<td>20 ( \mu g/mL )</td>
</tr>
<tr>
<td>Buffer</td>
<td>6.6±0.24</td>
</tr>
<tr>
<td>Abciximab, 0.1 ( \mu g/mL )</td>
<td>6.1±0.35</td>
</tr>
<tr>
<td>Tirofiban, 0.5 ng/mL</td>
<td>6.2±0.27</td>
</tr>
<tr>
<td>Eptifibatide, 0.01 ( \mu g/mL )</td>
<td>6.1±0.26</td>
</tr>
<tr>
<td>SC-54701, 1 nmol/L</td>
<td>6.5±0.13</td>
</tr>
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Results are expressed as geometric mean fluorescence intensity ± SD; \( n=3 \).

**Figure 4.** Analysis of fibrinogen binding to platelets during abciximab binding and dissociation. Platelets were incubated with or without abciximab for 30 minutes, then diluted 100-fold. At indicated time points, aliquots were removed and incubated with saturating concentration of FITC-abciximab for additional 10 minutes to identify unblocked receptors (\( \bullet \rightarrow \bullet \)). To test for presence of surface-bound fibrinogen, separate aliquots were incubated in parallel with FITC-labeled F26 for 10 minutes with (\( \bullet \rightarrow \bullet \)) or without (\( \circ \rightarrow \circ \)) stimulation by ADP/TRAP. Fibrinogen and abciximab binding to abciximab-treated platelets are expressed as percent of binding observed with control platelets incubated in parallel without abciximab. Data are representative of 3 experiments.

**Figure 5.** Formaldehyde “freezes” fibrinogen-binding–competent conformation of GP IIb/IIIa on eptifibatide-treated and tirofiban-treated but not abciximab-treated platelets. Platelets were fixed in presence of 10 \( \mu g/mL \) abciximab, 1 \( \mu g/mL \) eptifibatide, or 50 ng/mL tirofiban by the method of Peter et al \(^2\) and bound fibrinogen was detected with monoclonal antibody F26 (A) or by method of Du et al \(^15\) and fibrinogen binding assessed with FITC-fibrinogen (B). Data are average of 2 experiments.
P-selectin expression; (2) tirofiban and epifibatide can induce a fibrinogen binding-competent conformation of the GP IIb/IIIa receptor, but stable fibrinogen binding does not occur without fixation; (3) the slow off-rate of abciximab exposes only a small proportion of unblocked GP IIb/IIIa receptors at any time, and these also fail to stably bind fibrinogen; and (4) in experiments with unfixed, intact platelets, artificial thrombin generation may account for the fibrinogen binding reported by Peter et al. We therefore conclude that under physiological conditions, the intravenous GP IIb/IIIa antagonists currently in clinical use, as well as the oral GP IIb/IIIa antagonist xemilofiban, do not have an intrinsic activating property that results in platelet aggregation or stable fibrinogen binding to GP IIb/IIIa.

GP IIb/IIIa is known to undergo conformational changes when occupied by fibrinogen, RGD peptides, or RGD-peptide mimetics, resulting in exposure of ligand-induced binding sites.20–22 These occupancy-dependent conformational changes on intact, unfixed platelets were shown to be completely reversible, in that ligand-induced binding site epitopes became cryptic again on removal of RGD peptide.21,22 Du et al15 showed that if fixation was used to “freeze” the receptor in the occupied conformation (ie, preventing it from returning to a resting conformation) and the antagonist was removed, the receptor was then able to bind fibrinogen with high affinity. Our results (Figure 5) confirm these findings as well as those of Peter et al23; that is, platelets fixed in the presence of small-molecule GP IIb/IIIa antagonists are capable of binding fibrinogen after the antagonist is removed (Figure 5). Although abciximab may also induce GP IIb/IIIa to take on a fibrinogen-binding competent conformation that can be preserved by fixation,2 we were unable to reproduce this result (Figure 5) because we could not remove the abciximab after fixing the platelets, possibly because of methodological differences. In the absence of fixation, Peter et al23 found that low-dose abciximab added to PRP resulted, on stirring with added calcium and magnesium, in platelet aggregation, whereas we observed no aggregation under these same conditions (Figure 1). The reason for this difference is unclear. However, because Peter et al used citric acid for anticoagulation (of unstated concentration) whereas we used 3.2% sodium citrate, the calcium added according to Peter et al23 may have in some instances resulted in thrombin generation and subsequent platelet aggregation. In other experiments with native (unfixed) platelets, we reproduced the findings of Peter et al23 with respect to the biphasic pattern of fibrinogen binding that occurred in the presence of increasing concentrations of abciximab (Figure 3). However, additional controls in our experiments, for example, the addition of hirudin or the omission of calcium from the buffer, eliminated this pattern of fibrinogen binding. We therefore conclude that the fibrinogen binding was not GP IIb/IIIa antagonist induced but was an artifact of thrombin generation. In contrast, thrombin cannot explain the fibrinogen binding seen by Peter et al23 on abciximab-treated, GP IIb/IIIa–expressing Chinese hamster ovary (CHO) cells. However, GP IIb/IIIa expressed in a CHO cell background appears to behave differently from GP IIb/IIIa on intact platelets, because we show here (Figure 4) that dissociation of abciximab from intact platelets does not lead to stable fibrinogen binding. The presently demonstrated lack of fibrinogen binding across a wide range of antagonist concentrations (Figure 2, Figure 3 open bars, and Figure 4) suggests that the fibrinogen-binding—competent conformation of antagonist-occupied GP IIb/IIIa is rapidly reversed after the antagonist dissociates or that return of the receptor to a resting conformation may precede (and even possibly potentiate) antagonist dissociation.

Because the present results show that abciximab, epifibatide, tirofiban, and the active metabolite of xemilofiban do not, on dissociation, cause fibrinogen binding to GP IIb/IIIa, other explanations may need to be sought for the higher mortality rates observed in several trials of patients receiving low-dose oral GP IIb/IIIa antagonists1,3,5,7,9,11,12 and for the disappointing results of abciximab in the GUSTO-IV trial of unstable angina. For example, the untoward clinical outcomes of the oral RGD-mimetic GP IIb/IIIa antagonists may be related to the ability of RGD peptides to induce apoptosis, apparently by directly entering cells and inducing autoprocessing and enzymatic activity of procaspase-3.24

Conclusions

The findings of Peter et al2 that induction of fibrinogen binding and platelet aggregation is an intrinsic prothrombotic property of low-dose GP IIb/IIIa antagonists have been extensively referenced in the cardiology literature.1,3–12 However, the present study demonstrates by a number of independent methods that neither abciximab (the GP IIb/IIIa antagonist primarily studied by Peter et al),2 nor tirofiban, nor epifibatide (the other two FDA-approved GP IIb/IIIa antagonists), nor SC-54701 (the active metabolite of the oral GP IIb/IIIa antagonist xemilofiban) induce stable fibrinogen binding or platelet aggregation under physiological conditions. Furthermore, the findings of Peter et al2 in experiments with unfixed platelets are probably accounted for by artificial thrombin generation. Therefore, interpretations based on the results of Peter et al2 must be reconsidered.

References


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