Paradoxical Platelet Activation Was not Observed on Dissociation of Abciximab from GPIIb-IIIa Complexes

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Keywords

Platelet activation, GPIIb-IIIa complex, abciximab, antithrombotic therapy

Summary

The ability of abciximab to bind and dissociate from platelets raises the question of the conformational state of GPIIb-IIIa complexes losing abciximab and the risk of paradoxical drug-induced platelet activation. Platelets incubated with abciximab and mixed in vitro with c7E3 Fab-free platelets lost the drug to the new platelets giving a single platelet population with a unimodal abciximab distribution within 17 h. Precalibration of the receiving platelets with phycoerythrin-labeled anti-GPIIb monoclonal antibody (MoAb), permitted their identification by flow cytometry. Binding of PAC-1 and AP6, two MoAbs specific for activated GPIIb-IIIa, was then assessed to both losing and receiving platelet populations during transfer of abciximab. The subpopulation losing c7E3 Fab failed to show increased binding of these MoAbs. However, PAC-1 binding increased in both subpopulations after addition of ADP. Thus GPIIb-IIIa complexes are not in an activated state after dissociation of abciximab unless there is an additional source of activation.

Introduction

The GPIIb-IIIa complex (αIIbβ3 integrin) plays a central role in platelet aggregation and is the target of a new class of anti-platelet drugs (1). After platelet challenge by soluble agonists or following adhesion, GPIIb-IIa changes into an activated conformation, a process that involves ‘inside-out’ signaling (2). The activated state of GPIIb-IIa permits rapid high affinity binding of soluble adhesive proteins such as fibrinogen or von Willebrand factor and the formation of the interplatelet protein bridges that mediate platelet aggregation. Fibrogen binding to GPIIb-IIa involves multiple sites on both the adhesive protein and on the integrin (for a review, see 3). A primary fibrogen-binding site concerns the Ala299-Met314 sequence of GPIIIa which is recognized by the N-terminal dodecapeptide (γ400-411) of the fibrogen γ-chain (4). A second site is located on GPIIIa within a region between Asp109-Glu173 which is recognized by RGD-containing sequences within the fibrogen α-chain (5). Other RGD-containing proteins also bind to this site as presumably do small molecular mass RGD-containing peptides or peptidomimetics. These two sites represent allosterically interactive sites in the receptor (3).

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Abciximab (ReoPro®, Centocor, Malvern, PA) corresponds to Fab fragments of the chimeric form of the MoAb 7E3 where the murine constant region is replaced by an equivalent sequence for human IgG (reviewed in 1). PE-conjugated HIP1 is an anti-GPIbα MoAb from Pharmingen (Le Pont de Claix, France). PAC-1, a murine IgM MoAb to activated GPIIb-IIIa, was from Becton Dickinson Immunocytometry Systems (Le Pont de Claix, France). AP6, a murine IgM MoAb to fibrogen-bound GPIIb-IIIa, was kindly provided by Dr. TJ Kunicki (The Scripps Research Institute, La Jolla, CA). Fluorescein isothiocyanate (FITC)-conjugated Fab(′)1, fragments of a goat antibody to human IgG and FITC-conjugated F(ab′)2, fragments of a donkey antibody to mouse IgM were from Jackson ImmunoResearch (West Grove, Pennsylvania).

Materials and Methods

Antibodies

Abciximab (ReoPro®, Centocor, Malvern, PA) corresponds to Fab fragments of the chimeric form of the MoAb 7E3 where the murine constant region is replaced by an equivalent sequence for human IgG (reviewed in 1). PE-conjugated HIP1 is an anti-GPIbα MoAb from Pharmingen (Le Pont de Claix, France). PAC-1, a murine IgM MoAb to activated GPIIb-IIIa, was from Becton Dickinson Immunocytometry Systems (Le Pont de Claix, France). AP6, a murine IgM MoAb to fibrogen-bound GPIIb-IIIa, was kindly provided by Dr. TJ Kunicki (The Scripps Research Institute, La Jolla, CA). Fluorescein isothiocyanate (FITC)-conjugated Fab(′)1, fragments of a goat antibody to human IgG and FITC-conjugated F(ab′)2, fragments of a donkey antibody to mouse IgM were from Jackson ImmunoResearch (West Grove, Pennsylvania).
PA). Mouse IgM and IgG (Sigma, Saint-Quentin Fallavier, France) were used as controls.

Transfer of c7E3 Fab from one Platelet Population to another

Platelet-rich plasma (PRP) from human volunteers was prepared by centrifuging blood anticoagulated with ACD-A (Acid-Citrate-Dextrose NIH Formula A) at 120 g for 10 min. Typically, three 1 mL samples of PRP containing between 300,000 to 500,000 platelets/µL were then prepared. The first sample was incubated with abciximab (at 1, 5 or 10 µg/mL, see text) for 15 min at room temperature (sample A). The second sample was labeled with PE-conjugated anti-GPIIb/IIIa MoAb at a dilution of 1/50 (v/v) under the same conditions in the dark (sample B). The third sample was incubated without added antibody or with control mouse immunoglobulin (sample C). To eliminate free antibody, samples were then centrifuged at 1000 g for 10 min, in the presence of 25 µg/mL apyrase (Sigma), 100 nM prostaglandin E1 (PGE1) and 1 volume ACD-A for 9 volumes of PRP. The platelet pellets were carefully resuspended in Tyrode buffer (137 mM NaCl, 2 mM KCl, 0.3 mM NaH₂PO₄, 1 mM MgCl₂, 5 mM Hepes, 12 mM NaHCO₃, 5.5 mM glucose, 0.1% (wt/vol) bovine serum albumin (BSA), pH 7.3, and the platelet count adjusted to 300,000/µL or higher depending on the experiment. To study the dissociation of abciximab

![Image](image_url)

**Fig. 1** Transfer of abciximab between platelet populations. A volume of abciximab-bearing platelets was added to an equal volume of antibody-free platelets and the mixture incubated at room temperature. Samples were taken after the initial mixing (T1), after 4 h and after 17 h and incubated with FITC-labeled anti-human IgG. The surface distribution of abciximab was then assessed by flow cytometry. A progressive transfer of abciximab from the losing (L) to the receiving population (R) gave rise to a homogeneously labeled population of platelets with a unimodal abciximab distribution. When present, the horizontal bar identifies the population in the mixture included in the calculation of the MFI. Also shown are the histograms obtained for the abciximab-free and the abciximab prelabeled platelets incubated alone.

<table>
<thead>
<tr>
<th>1 µg/ml</th>
<th>c7E3 Fab alone</th>
<th>L</th>
<th>R</th>
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<tbody>
<tr>
<td>T1</td>
<td>126 ± 63</td>
<td>74.6 ± 24</td>
<td>7.5 ± 1.2</td>
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<tr>
<td>T2</td>
<td>106 ± 14</td>
<td>72.3 ± 22</td>
<td>10 ± 2.3</td>
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<tr>
<td>T3</td>
<td>94.3 ± 6</td>
<td>58.3 ± 8</td>
<td>10.8 ± 5.2</td>
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Table 1

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<tr>
<th>5 µg/ml</th>
<th>c7E3 Fab alone</th>
<th>L</th>
<th>R</th>
</tr>
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<tbody>
<tr>
<td>T1</td>
<td>260.6 ± 75</td>
<td>187.7 ± 88</td>
<td>13.2 ± 6</td>
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<tr>
<td>T2</td>
<td>260 ± 104</td>
<td>179.6 ± 93</td>
<td>30.3 ± 14.8</td>
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<tr>
<td>T3</td>
<td>266 ± 108</td>
<td>140 ± 67</td>
<td>33.1 ± 13.8</td>
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MFI expressed as the mean of 3 experiments (with standard deviation). Platelets preincubated with c7E3 Fab were separated from free antibody and mixed with abciximab-free PE-anti-GPIIb/IIIa-labeled platelets at a ratio of 1:2. Bound abciximab was measured in both L and R populations by flow cytometry at T1 (immediately after mixing), T2 (at 2h) and T3 (4h).
Platelets incubated or not with abciximab

<table>
<thead>
<tr>
<th></th>
<th>No c7E3 Fab</th>
<th>c7E3 Fab 1 µg/ml</th>
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<tr>
<td>% activated platelets</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>T1</td>
<td>10</td>
<td>10</td>
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<tr>
<td>T2</td>
<td>20</td>
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<td>T3</td>
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Mixture of L + R populations

Flow cytometric detection of activated platelets binding PAC-1 before and after interplatelet transfer of abciximab. Platelets were preincubated with 1 µg/mL abciximab, separated from free drug, and mixed with a twofold excess of PE-anti-GPIb-labeled receiving platelets. Samples were analyzed at T1 (immediately after mixing), T2 (at 2 h) and T3 (at 4 h). A) Percentage of activated platelets in abciximab-free control populations. B) Percentage of activated platelets in samples preincubated with 1 µg/mL of abciximab. C) Dot plot representation at T2 of the mixture of platelets (L stands for the c7E3 Fab-bearing platelet population losing abciximab and R is the initially c7E3 Fab-free platelet population that receives abciximab). The R population is identified by a high MFI in FL2. Activated platelets that have bound PAC-1 in the L and R subpopulations are in the upper quadrants. D) The percentage of activated platelets in the L and R populations at T1, T2 and T3 shows that transfer of abciximab does not lead to increased PAC-1 binding.

Assessment of Platelet Activation

To determine if the loss of c7E3 Fab from GPIIb-IIIa left the integrin in an activated state, we examined the binding of PAC-1 and AP6. Binding of these antibodies was evaluated after mixing the platelet populations as described above (T1), and then after 2 and 4 h incubations at room temperature (T2 and T3). Platelet activation was evaluated in samples A, B and C alone as well as in the mixture. Briefly, a volume (10 µL) of platelet suspension was incubated with 100 µL of FITC-conjugated anti-human IgG (1/100) for 15 min in the dark at room temperature prior to analysis by flow cytometry as described (12, 13). The platelet population receiving abciximab was identified in the mixture by the presence of the PE-conjugated anti-GPIb antibody. The irreversibility of the binding of this intact MoAb to platelets was confirmed in preliminary experiments performed by mixing equivalent volumes of samples B and C, no transfer was observed (data not shown).

Results

Transfer of Abciximab

The transfer of c7E3 Fab was first studied from platelets preincubated with 10 µg/mL abciximab and mixed with an equal number of abciximab-free platelets (Fig. 1). Separate histograms were present at T1, although already there is a marked decrease in the MFI of the L
population. Although T1 represents the initial mixing, it also includes the time taken for the incubation with the FITC-anti-human IgG antibody recognizing the humanized domain of c7E3 Fab (12). As the incubation continued, the valley between the peaks filled and the peaks closed until a single histogram showing a unimodal distribution of abciximab was obtained at about 17 h. To be certain of identifying the R population, the latter were subsequently prelabeled with the PE-anti-GPIb-MoAb. We next compared the transfer of abciximab from platelets preincubated with either 1 μg/ml or 5 μg/ml of this drug to the R population. The proportion of platelets in the R population was also increased to 2:1. Transfer of abciximab was more rapid under these conditions. It was not affected by the presence of the anti-GPIb MoAb which itself did not transfer from one platelet to another (data not shown). Movement of c7E3Fab to the R population was seen at both abciximab concentrations with, as expected, the MFI of the R population increasing faster at 5 μg/ml abciximab. A quantitative assessment of the changes in MFI as seen for 3 donors is given in Table 1.

Platelet Activation Markers

After having confirmed that abciximab does indeed transfer rapidly to newly added platelets, we next evaluated the binding of PAC-1 and AP6 to platelets losing abciximab. Figure 2 illustrates the results for platelets preincubated with 1 μg/ml abciximab. As shown for 3 donors in panel A, the mean percentage of platelets binding PAC-1 in the control sample processed without preincubation with abciximab was 18.3% ± 2.7 compared to values of < 2% with control IgM, suggesting that some activation had occurred during the centrifugation and resuspension steps (see Methods). At T2, the value was 16.7% ± 2.5 and at T3 it was 12% ± 7. In panel B, samples were preincubated with c7E3 Fab but not mixed and the percentage of activated platelets at T1, T2 and T3 was lower at 11.2% ± 5.9, 7.3% ± 4.4, and 2.8% ± 2.2, respectively showing that even at this nonsaturating concentration abciximab was inhibiting expression of the PAC-1 epitope. In panel C we illustrate a dot-plot corresponding to the platelet mixture analyzed at T2 with FITC-fluorescence (PAC-1 binding) on the y axis and PE fluorescence (PE-anti-GPIbα) on the x axis. The R population detected by the PE fluorescence was exclusive to quadrants 3 and 4 and was well separated from the L population in quadrants 1 and 2. Platelets positive for PAC-1 are present in the upper quadrants (2 and 3). Panel D shows the mean values for the 3 donors of PAC-1 positive platelets in the L and R populations at T1, T2 and T3. In the L population, where c7E3 Fab is leaving the platelets, the percentage of platelets binding PAC-1 was 4.2% ± 3.3 at T1, 3.4% ± 0.7 at T2 and only 1% ± 0.34 at T3. Thus activation tended to decrease rather than increase. Analysis at T2 and T3 of platelets in sample B alone, or after dilution of sample B with sample C as the control, showed that the presence of the anti-GPIbα MoAb had no effect on the expression of the PAC-1 epitope (results not shown).

Experiments were repeated after preincubation with 5 μg/mL abciximab. Few of the abciximab-treated platelets now bound PAC-1 at T1, T2 and T3 (values of 1.8 ± 2.7, 2.1 ± 2, and 0.5 ± 0.2 respectively). These results clearly show that c7E3 Fab at this higher dose either prevented platelet activation and/or blocked PAC-1 binding. During transfer, the quantitative analysis of PAC-1 binding confirmed that in the L population this remained at close to baseline levels (1.3 ± 2.2, 1.8 ± 1.6, 2.1 ± 1.5, at T1, T2 and T3 respectively). In the R population, the amount remained variable in this series.

Binding of AP6 was also studied. Figure 3 illustrates typical results for platelets which had been preincubated with 1 μg/mL of abciximab. Again, the percentage of platelets positive for AP6 had typically fallen at T1 from 11% to 4%, showing a protective effect of abciximab (not shown). In the platelet mixture, there were 5% (T1) and 1.2% (T2) platelets positive for AP6 in the L population, 12% (T1) and 2.5% (T2) in the R population. The results for AP6 at T2 and T3 (not illustrated) compared well with those obtained for PAC-1 for both doses of abciximab. Significantly, although the binding of fibrinogen to GPIIb-IIIa induces the LIBS (‘Ligand-induced binding site’) epitope recognized by AP6 (11), the presence of abciximab did not.

We finally looked at the capacity of abciximab-treated platelets to be activated by 5 μM ADP at T2 (Fig. 4). After stimulation, the number of activated platelets binding PAC-1 in the L population increased from 3.4% to 43% and from 11.3% to 56% in the R population (values for a
Discussed has been reported that on dissociation from platelets abciximab leaves the GPIIb-IIIa complex in an active conformation able to bind fibrinogen (7). A latent activating potential of abciximab could potentiate thrombotic risk at the end of treatment when circulating levels of the drug are insufficient to block fibrinogen binding to the receptor. In their study, Peter et al. (7) used a ‘washout’ procedure in which, after incubation with abciximab, platelet-rich plasma was fixed with paraformaldehyde prior to washing platelets in a large excess of fibrinogen-containing buffer. We have studied this phenomenon using a less drastic procedure, taking advantage of an observation first reported by Christopoulos et al. (8) who showed that mixing platelets with prebound c7E3 Fab and an antibody-free platelet population promoted antibody interchange. Rapid rebinding of abciximab is a function of the high affinity of c7E3 Fab for the integrin (9). By mixing together platelets losing abciximab (L population) and platelets receiving abciximab (R population) prelabeled with an anti-GPIb MoAb, we were able to use flow cytometry to detect activated integrin in both populations without further washing.

We first confirmed that the kinetics of transfer of c7E3 Fab from the L to the R populations was dependent on the size of the R population and on the concentration of c7E3 Fab during the initial preincubation of the platelets. A rapid initial decrease in the MFI of the L population deserves comment. Although platelets were separated from free abciximab, loss of unbound c7E3 Fab absorbed within the open surface canalicular system is one possible explanation. Trafficking of abciximab within platelets and transfer to internal membrane pools has been demonstrated (14-16). Nevertheless, although abciximab has been localized to α-granules, it does not accumulate in them and once an equilibrium has been attained the proportion of surface-bound drug relative to that inside the platelet remains constant suggesting continued receptor cycling (14). Thus internalization and storage of abciximab is unlikely to be an explanation for surface loss, which was not seen in the absence of the R population. An alternative mechanism requiring confirmation is that platelet to platelet contact facilitates initial transfer. Our finding could have clinical significance as platelet transfusion in the event of bleeding will promote abciximab interchange as well as provide hemostatically active platelets. Platelet function starts to be restored as soon as the degree of receptor occupancy falls below the 80% level necessary for maximal inhibition of ADP-induced platelet aggregation (data reviewed in 17).

Platelets losing c7E3 Fab clearly did not bind PAC-1 present in the medium. These results unambiguously show that GPIIb-IIIa was not left in an activated conformation. We used two doses of abciximab, doses selected to mimic abciximab loss from platelets of patients that have been treated clinically with currently used bolus plus infusion regimens (9, 15, 18). One explanation for the difference between our results and those of Peter et al. (7) is that their use of paraformaldehyde fixation prevented a spontaneous return to a normal conformation of GPIIb-IIIa. Another possibility is that the additional experimentation used by these authors resulted in secondary changes affecting the activation status of the integrin.

PAC-1 recognizes discontinuous binding sites on both GPIIb and GPIIIa, whereas indirect evidence implicates binding of abciximab to GPIIIa (6). Our results confirm that abciximab blocks the binding of PAC-1 to activated GPIIIa. We have previously shown that although patients with coronary artery disease may possess elevated levels of activated platelets, these levels fall or are abolished after administration of abciximab (13). In the same study, activated platelets recognized by PAC-1 reappeared in the circulation in the days following the stopping of the abciximab infusion. However, considerable interpatient variation was noted both in the timing of their reappearance and in their abundance (unpublished data). Here, we show that addition of ADP at T2 in the transfer experiments resulted in many activated platelets in both the L and R populations. Thus platelets having received and lost abciximab are not refractory to stimulation. As abciximab blocks GPIIb-IIIa function but does not eliminate the cause of ongoing platelet activation in coronary artery disease, the use of follow-up therapy with another anti-platelet drug such as clopidogrel may have a rational basis (19).

AP6, specific for an epitope contained in the 209-222 aa sequence of GPIIIa binds to GPIIb-IIIa on activated platelets only when the latter are occupied by fibrinogen. It was used here as an indirect measure of fibrinogen binding and of GPIIb-IIIa activation. Although PAC-1 is widely used for measuring platelet activation, fibrinogen may recognize additional binding sites in the ligand-binding pocket (see 2). In fact, our results with AP6 were similar to those obtained for PAC-1 and no evidence was obtained that dissociation of abciximab left GPIIb-IIIa in a conformation able to bind fibrinogen. The epitope recognized by AP6 is an anti-LIBS (11). Interestingly, abciximab binding did not substitute for fibrinogen in inducing the expression of the AP6 epitope. The domain recognized by abciximab on GPIIIa requires aa 179-183 (6), close enough to the epitope recognized by AP6 to suggest steric interference if abciximab binds directly to this sequence. Nevertheless, abciximab has been shown to induce other LIBS epitopes on GPIIb-IIIa showing that the integrin does undergo some conformational change after its binding (20, 21). However, although GPIIb-IIIa may have a dynamic structure, our results show that abciximab does not leave it in an irreversibly activated conformation.

It should be underlined that our results apply only to abciximab and should not be extrapolated to other small molecular mass inhibitors of GPIIb-IIIa which have to be judged individually. Small molecular mass anti-GPIIb-IIIa drugs also induce LIBS expression on binding to the integrin. Eptifibatide and tirofiban are good examples (20). One compound, XP280, induced long-lived conformational changes resistant to protein denaturation (22). Others have classified the effect of anti-GPIIb-IIIa drugs on receptor conformation by using a commercially available kit containing MoAbs 1 and 2 whose binding are assessed in flow cytometry. Thus, eptifibatide and the oral anti-GPIIb-IIIa antagonists, orbifiban and xemilofiban, all displaced the binding of MoAb 2 (23-25). The site recognized by MoAb 2 was termed a ligand-attenuated binding site. In contrast, tirofiban and lamifiban inhibited both MoAb 1 and MoAb 2 binding while abciximab inhibited MoAb 1 binding but had no effect on MoAb 2 (23). Such results with abciximab are compatible with steric inhibition of the binding of MoAb 1 (an inhibitor of fibrinogen binding) while the normal binding of MoAb 2 would suggest an unchanged conformation of the region on GPIIIa recognised by this antibody (23). There is no evidence from major clinical trials of an upsurge of thrombotic events in the days following the stopping of abciximab therapy, indeed the benefits of abciximab are maintained.
over 30 days and, in some reports, for even longer (data reviewed in 26). Such results back up our findings and suggest that abciximab therapy is unlikely to be an active cause of thrombotic complications in the days following the termination of c7E3 Fab infusion.

References


