Ability of Anti-Glycoprotein IIb/IIIa Agents to Dissolve Platelet Thrombi Formed on a Collagen Surface Under Blood Flow Conditions

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**OBJECTIVES** We examined the lytic effects of anti-glycoprotein (GP) IIb/IIIa agents on platelet thrombi formed on the collagen surface under blood flow conditions.

**BACKGROUND** Anti-GP IIb/IIIa agents may influence platelet thrombi already formed.

**METHODS** Blood samples were anticoagulated either by the specific antithrombin Argatroban (100 µM) or by unfractionated heparin (0.1 U/ml). After platelet thrombi were formed on a collagen surface following 6-min perfusion of whole blood obtained from eight adult donors containing fluorescinated platelets at a wall shear rate of 1,500 s⁻¹, additional blood samples from the same donors either containing or not containing anti-GP IIb/IIIa agents (abciximab, eptifibatide, or tirofiban) were perfused on these thrombi. The three-dimensional structures of the platelet thrombi were continuously observed by laser confocal microscopy equipped with a piezo-electric motor control unit and recorded.

**RESULTS** The platelet thrombi started to dissolve after perfusion of blood containing the anti-GP IIb/IIIa agents, whereas their growth resumed after subsequent perfusion of control blood. Only a single layer of platelets having heights of 3 ± 1 µm, 3 ± 2 µm, and 3 ± 1 µm, respectively, could be seen after 6-min perfusion of blood containing abciximab, eptifibatide, and tirofiban, whereas the initial height of the platelet thrombi of 8 ± 2 µm increased to 11 ± 4 µm after subsequent perfusion of control blood (n = 8). The volume of the platelet thrombi, which was 3,352 ± 1,045 µm³ before starting the second perfusion, was reduced to 778 ± 102 µm³, 812 ± 122 µm³, and 856 ± 144 µm³ after 6-min perfusion of blood containing abciximab, eptifibatide, and tirofiban, respectively.

**CONCLUSIONS** We have shown in this study that anti-GP IIb/IIIa agents possess the ability to dissolve platelet thrombi. (J Am Coll Cardiol 2004;44:316–23) © 2004 by the American College of Cardiology Foundation

It is now well known that agents blocking the platelet glycoprotein (GP) IIb/IIIa receptor (integrin αIIbβ3) reduce the incidence of early thrombotic complications after angioplasty (1–8) and prevent death and myocardial infarction in patients with acute coronary syndromes (8). Anti-glycoprotein IIb/IIIa agents have been reported to inhibit platelet aggregation induced by chemical activation (9,10) or by shearing (11), and to inhibit platelet thrombus formation in animal models of endothelial damage (12). In addition to these effects, several previous clinical and animal studies have reported that the anti-GP IIb/IIIa agents enhance the reperfusion rate after administration of fibrinolytic agents and may even induce spontaneous reperfusion (13–18), although the effect was not marked when the dose of fibrinolytic agent was reduced to half (19). These findings led us to wonder whether anti-GP IIb/IIIa agents might also have the potential to augment thrombolysis or induce thrombolysis by themselves, in addition to their well-known preventive effect on thrombus formation.

Previously, platelet aggregation induced by chemical agonists, such as adenosine diphosphate or thrombin, was reported to disaggregate when the binding capacity of GP IIb/IIIa was affected (20,21). However, this experimental finding may or may not be relevant to the question of enhanced thrombolysis induced by these agents in vivo, because the mechanism of platelet thrombus formation in vivo, especially under high shear stress conditions, might not be the same as that underlying platelet aggregation in vitro (22,23). Multiple receptor-ligand interactions, including von Willebrand factor (VWF) binding to both GP Ibα and GP IIb/IIIa, are involved in the former (22,23), whereas the latter is exclusively mediated by fibrinogen binding to activated GP IIb/IIIa (9). In the present study, we found that the anti-GP IIb/IIIa agents available for clinical use, namely, abciximab, eptifibatide, and tirofiban, not only inhibit platelet thrombus formation on a collagen surface under blood flow conditions but also have the potential to dissolve platelet thrombi already formed on the surface. These results may explain why these anti-GP
IIb/IIIa agents augment the thrombolytic effects of fibrinolytic agents or induce spontaneous reperfusion by themselves.

**METHODS**

**Sample preparation.** The anti-GP IIb/IIIa agents used in this study were abciximab (ReoPro; Centocor, Malvern, Pennsylvania), eptifibatide (Integrillin; Cor Therapeutic, Inc., San Francisco, California) and tirofiban (Aggrastat; Merck & Co., Inc., West Point, Pennsylvania), all of which are available for clinical use in many countries (24). Venous blood from eight normal volunteers abstaining from any type of medication was drawn through 19-G needles into plastic syringes containing one-tenth of their volume of the specific thrombin inhibitor Argatroban (Mitsubishi Kagaku, Tokyo, Japan) (25) or by commonly used anticoagulant of heparin. The final concentrations of the anticoagulant used were 100 μM for Argatroban and 0.1 U/ml for heparin. Argatroban, instead of the more commonly used anticoagulant citrate, was used for anticoagulation, to avoid pleiotropic effects through decreased cation concentration. Heparin, although used commonly in the clinical setting, was used only to show the relevance of the results obtained with Argatroban, because it may influence the results by either activating the platelets or modifying the interaction between VWF and GPIbα (22). Platelets in whole blood were rendered fluorescent by the addition of mepacrine (Sigma Co. Ltd., St. Louis, Missouri), according to a previously established procedure (25).

**Preparation of the flow chamber and visualization of the platelet thrombi.** Acid-insoluble fibrillar type I collagen from bovine Achilles tendon (Sigma Co.) was immobilized on a glass coverslip (Corning Inc., Acton, Massachusetts; 24 mm × 50 mm) in a parallel-plate flow chamber (25). The distance between the two glass plates was fixed at 220 μm by the placement of a silicon gasket. Then, the blood samples were introduced into the chamber with a syringe pump (Harvard Apparatus Co. Ltd., Holliston, Massachusetts) at a constant flow rate to achieve a wall shear rate of 1,500 s⁻¹. Platelet thrombi forming on the surface of collagen were visualized with an inverted stage epifluorescence video-microscope system equipped with a 480-nm excitation light source (DM IRB, IRB-FLUO; Leica, Wetzlar, Germany) (25). The microscopic images were digitized online with a photosensitive color charge-coupled device camera (L-600; Leica) and stored as digital images in a personal computer (Power Macintosh G4; Apple Co. Ltd., Palo Alto, California). To quantify the percentage surface area coverage by the platelets, the digital color images were converted into black-and-white images using the National Institutes of Health (NIH) Image software (public domain software by Dr. Wayne Rasband, NIH, version 1.62), and the percentage surface area coverage by the platelets was calculated.

To detect the effects of anti-GP IIb/IIIa agents of dissolving platelet thrombi formed on the collagen surface, three-dimensional structural analysis was conducted using an ultra-fast laser confocal microscope equipped with a piezo-electric motor control unit (Fig. 1A). Using a confocal unit composed of a rapidly rotating disk having 20,000 pinholes and micro lenses on it (CSU10; Yokogawa Medical Co., Tokyo, Japan), each confocal image could be obtained within 10 ms (26). To visualize the three-dimensional structure of the platelet thrombi formed on the collagen surface, the objective lenses were up and down (20 μm/50 s) at a constant speed controlled by a piezo-electric motor control system, so that scanning images of the thrombi were obtained. The confocal images were enhanced using an image intensifier (SRUB GEN III +, Solam, Salt Lake City, Utah, and Intermedical Co., Tokyo, Japan), and the intensified images were stored in a digital video recorder (Handycum; Sony Co., Tokyo, Japan) and transferred to a personal computer (Power Macintosh G4, Apple Co. Ltd.).

Three-dimensional projection images of the thrombi were obtained using shareware NIH images, as previously reported (26). For quantification, the cross-sectional area covered by platelets was calculated at the base and 3, 6, and 9 μm above the base of the platelet thrombi. The results were expressed as a percentage of the area covered at the base of the thrombi. The volume of the platelet thrombus was calculated by e-section integration of the cross-sectional area in each micrometer.

**Experimental protocol.** Experiments were performed as described in Figure 1B and its legend. Briefly, 15 ml of blood samples either containing or not containing anti-GP IIb/IIIa agents at concentrations sufficient enough to inhibit platelet thrombus formation on the collagen surface (abciximab: 10 μg/ml; eptifibatide and tirofiban: 0.5 μM) was perfused on the same collagen surface on which platelet thrombi were already formed by the initial 15 ml of control blood perfusion. The second perfusion, of blood either containing or not containing the anti-GP IIb/IIIa agents, was started immediately after the first perfusion was completed, without stopping the blood flow, to avoid the falling of leukocytes onto the platelet thrombi because of gravity, as it is speculated that the presence of leukocytes may influence the stability of the platelet thrombi. The three-dimensional structures of the platelet thrombi were assessed every 50 s by the three-dimensional imaging technique described earlier. All the experiments were performed at room temperature, controlled between 22°C to 26°C.

**Statistical analysis.** All numerical results were presented as mean ± SD, unless otherwise stated. The effects of various concentrations of the three anti-GP IIb/IIIa agents under study on the percentage surface area coverage by the

**Abbreviations and Acronyms**

- GP = glycoprotein
- NIH = National Institutes of Health
- VWF = von Willebrand factor

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platelets were tested by two-way analysis of variance. The effects of the second perfusion of blood containing one of the various anti-GP IIb/IIIa agents on the volume and maximum height of the platelet thrombi already formed were also tested by one-way analysis of variance. The differences between groups of data were assessed by Newman-Keuls test. A p value of \( < 0.05 \) was considered to denote statistical significance.

RESULTS

Effect of anti-GP IIb/IIIa agents on platelet thrombus formation on the collagen surface. All the anti-GP IIb/IIIa agents tested in our study inhibited platelet thrombus formation on the collagen surface in a dose-dependent manner no matter whether blood was anticoagulated with Argatroban or heparin (Fig. 2). There were no statistically significant differences between the effects of abciximab and eptifibatide, or between those of eptifibatide and tirofiban. On the other hand, the percentage surface area coverage of 7.7 ± 2.6% achieved with tirofiban at a dose yielding the maximum inhibitory effect (0.5 \( \mu \text{M} \)) was significantly higher than that of 4.6 ± 2.2% achieved with the maximum inhibitory dose of abciximab (10 \( \mu \text{g/ml} \); p < 0.05). Only one layer of attached platelets, without z-section growth mediated by platelet cohesion, was observed after perfusion of blood containing abciximab (10 \( \mu \text{g/ml} \)), eptifibatide (0.5 \( \mu \text{M} \)), or tirofiban (0.5 \( \mu \text{M} \)), whereas three-dimensional growth mediated by platelet cohesion was observed in the absence of these agents (Fig. 3).

Effect of anti-GP IIb/IIIa agents on platelet thrombi formed on the collagen surface. Soon after blood containing any one of the anti-GP IIb/IIIa agents began to be perfused over the collagen surface where platelet thrombi had formed, the thrombi started to dissolve no matter whether blood was anticoagulated with Argatroban or heparin (Fig. 2). There were no statistically significant differences between the effects of abxiximab and eptifibatide, or between those of eptifibatide and tirofiban. On the other hand, the percentage surface area coverage of 7.7 ± 2.6% achieved with tirofiban at a dose yielding the maximum inhibitory effect (0.5 \( \mu \text{M} \)) was significantly higher than that of 4.6 ± 2.2% achieved with the maximum inhibitory dose of abciximab (10 \( \mu \text{g/ml} \); p < 0.05). Only one layer of attached platelets, without z-section growth mediated by platelet cohesion, was observed after perfusion of blood containing abciximab (10 \( \mu \text{g/ml} \)), eptifibatide (0.5 \( \mu \text{M} \)), or tirofiban (0.5 \( \mu \text{M} \)), whereas three-dimensional growth mediated by platelet cohesion was observed in the absence of these agents (Fig. 3).
anti-GP IIb/IIIa agents. Not only single platelets, but also masses of platelet thrombi became detached from the body of the thrombi when blood containing an anti-GP IIb/IIIa agent was perfused. Three-dimensional analysis of the thrombi revealed that both the height and the volume of the platelet thrombi began to decrease owing to dissolution of platelet cohesion (Figs. 4 and 5). The maximum height as well as the volume of the platelet thrombi immediately before and after completion of perfusion of blood containing one of the anti-GP IIb/IIIa agents, abciximab (10 μg/ml), eptifibatide (0.5 μM), or tirofiban (0.5 μM), are summarized in Table 1. Indeed, the maximum height of the thrombi and their volume decreased significantly after 6-min perfusion of blood containing one of the anti-GP IIb/IIIa agents.

Three-dimensional imaging revealed only a single layer of attached platelets was seen in the presence of any of the anti-GP IIb/IIIa agents tested (10 μg/ml abciximab, 0.5 μM eptifibatide, and tirofiban). The results shown are representative of the results of eight replicated experiments. The results with eptifibatide were similar to those obtained with abciximab and tirofiban, but are not shown because of space limitation. There were no differences in the results when the blood was anticoagulated by heparin. For the accompanying videos corresponding to Figure 3 (Videos 1, 2, and 3), please see the July 21 issue of JACC at www.cardiosource.com/jacc.html.

**DISCUSSION**

Anti-GP IIb/IIIa agents were developed as antiplatelet agents, based on the premise that they would block platelet aggregation (7–9). Clinical experiences, as well as animal experiments, have demonstrated that early reperfusion of coronary arteries can be induced by the administration of anti-GP IIb/IIIa agents, regardless of whether or not fibrinolytic agents were also administered concomitantly (13–19). We showed that the widely used GP IIb/IIIa antagonists abciximab, eptifibatide, and tirofiban have the potential to dissolve platelet thrombi formed on a collagen surface. We postulate that the effect of these agents of dissolving platelet thrombi already formed, in addition to their preventive effect on de novo platelet thrombus formation, contributes to the reportedly augmented thrombolytic effects observed when these agents are administered in combination with fibrinolytic agents, and to the increased rate of reperfusion achieved when these agents are used alone (13–19).

Unlike in the case of platelet aggregation induced by chemical agonists, which is known to be mediated exclusively by fibrinogen binding to activated GP IIb/IIIa, platelet thrombus formation on the collagen surface requires stimulation of several platelet receptors induced by their binding of the corresponding ligands, including the GP Ibα-VWF interaction (27–29), GP IIb/IIIa ligation by plasma proteins including fibrinogen and VWF (27–29), P2Y1 and P2Y12 stimulation by adenosine diphosphate released from activated platelets (30,31), are involved in
platelet cohesion under high shear stress conditions. Although numerous receptor-ligand interactions, such as the initial tethering mediated by VWF-GP Ib/H9251 interaction, may play a role in the formation of platelet thrombi, our present findings suggest that GP IIb/IIIa ligation is required for stable platelet cohesion because only platelet bound with platelet, but not with the matrix surface was susceptible to the second perfusion of blood containing one of the anti-GP IIb/IIIa agents. Further studies using our assay system, including the clarification of the relative importance of other factors, such as CD40 ligand (32) and P-selectin (33), are under way.

**Possible mechanism.** We propose the following mechanism to explain how platelet thrombi are dissolved by anti-GP IIb/IIIa agents (Fig. 6); however, please note that the mechanism shown in Figure 6 is purely speculative. First, we speculate that the binding of crucial ligands, whether VWF or fibrinogen, is reversible; therefore, ligand molecules can be replaced by anti-GP IIb/IIIa agents when thrombi are perfused with blood containing these agents. Platelets begin to get detached from the thrombi when the

**Figure 4.** Effect of anti-glycoprotein (GP) IIb/IIIa agents on the dissolution of platelet thrombi formed on the collagen surface. The experiments were performed as described in the legend for Figure 1B. Blood anticoagulated with Argatroban either containing or not containing anti-GP IIb/IIIa (abciximab: 10 μg/ml, eptifibatide: 0.5 μM, tirofiban 0.5 μM) was perfused on the collagen surface on which platelet thrombi had already formed as a result of previous perfusion of control blood. The three-dimensional projection images of the platelet thrombi (the projection images from the top [A], 45° position from the x axis [B], and the side [C]) before and after the second perfusion of blood containing (II) or not containing one of the anti-GP IIb/IIIa agents of abciximab (I) are shown in the left and right panel of I and II. The results are representative of those of eight replicated experiments. No differences in effects were observed among the three anti-GP IIb/IIIa agents tested. No differences in the effects of anti-GP IIb/IIIa agents were observed when blood anticoagulated with heparin was used. For the accompanying videos corresponding to Figure 4 (Videos 4, 5, 6, and 7), please see the July 21 issue of JACC at www.cardiosource.com/jacc.html.

**Figure 5.** Effect of anti-glycoprotein (GP) IIb/IIIa agents on platelet thrombi formed on the collagen surface. The experiments were performed in a manner similar to that described in the legend for Figures 1 and 4. The three-dimensional structure of the platelet thrombi formed on the collagen surface after 6-min perfusion of blood on the collagen surface at 1,500 s⁻¹ was quantified by calculating the cross-sectional areas of the platelet thrombi using the National Institutes of Health image software at every 3 μm from the collagen surface. The results are expressed as a percentage of the largest cross-sectional area obtained at the collagen surface level. The results shown are the mean and standard error of eight replicated experiments. Similar results were obtained in the condition when blood was anticoagulated by heparin.
crucial number of ligand molecules necessary to maintain the integrity of the thrombi is replaced by anti-GP IIb/IIIa agents, and the power generated by the binding of ligands to GP IIb/IIIa is no longer sufficient to resist the shear force generated by blood flow. Other adhesive proteins, including P-selectin (33) and CD40 ligand (32), may be involved in stabilizing platelet thrombi, but their roles were not investigated in the present study.

**Advantages and limitation of our methods.** Our newly developed three-dimensional analysis system depended on the unique features of the confocal microscopy system (26). However, there were still obvious methodological limitations in our assay system, especially when seeking to apply the experimental results to understand the events in vivo. Obviously, we could not reproduce the complex in vivo arterial flow conditions (34) in our flow chamber system. Under the complex flow conditions prevailing in vivo, including low, high, or changing shear rate conditions, the ability of anti-GP IIb/IIIa agents to dissolve platelet thrombi might be greater or lesser than that shown in our experiments. We also conducted the experiments at room temperature rather than at body temperature (37°C), mostly because the thrombi were more prominent, and the volume of blood necessary for thrombus formation was smaller at room temperature (28). Nevertheless, the important message that anti-GP IIb/IIIa agents can cause dissolution of platelet thrombi by adversely affecting platelet cohesion is not likely to be influenced by these methodological limitations.

One might argue that the ability of anti-GP IIb/IIIa agents to dissolve platelet thrombi in vivo might be weaker than that shown in our experiment, because fibrin formation, which plays a crucial role in stabilizing thrombi, was inhibited under our study conditions. The mass of platelet thrombi detached from the body of the platelet thrombi we have detected herein, might also be unstable without fibrin.

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**Table 1.** Effects of Subsequent Perfusion of Blood Containing One of the Anti-GP IIb/IIIa Agents on the Height and the Volume of the Pre-Existing Platelet Thrombi

<table>
<thead>
<tr>
<th></th>
<th>Before the Second Perfusion†</th>
<th>After the Second Perfusion‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control‡</td>
<td>Abciximab</td>
</tr>
<tr>
<td>Height of thrombi (µm)</td>
<td>8 ± 2</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>Volume of thrombi (µm³)</td>
<td>3,352 ± 1,045</td>
<td>7,450 ± 1,825</td>
</tr>
</tbody>
</table>

*The height and the volume of the platelet thrombi before and after the second perfusion of blood containing or not containing abciximab (10 µg/ml), eptifibatide (0.5 µM), or tirofiban (0.5 µM) are shown. †Values immediately before the commencement of the second perfusion. ‡After completion of the second perfusion of blood not containing any of the anti-GP IIb/IIIa agents. §Values after completion of the second perfusion of blood containing any one of abciximab, eptifibatide, or tirofiban. ‡Indicates that the difference compared with the value determined before the second perfusion was significant.

GP = glycoprotein.

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**Figure 6.** Speculative mechanism explaining the thrombus-dissolving effects of anti-glycoprotein (GP) IIb/IIIa agents. This figure summarizes the possible mechanism by which anti-GP IIb/IIIa agents dissolve platelet thrombi formed on a collagen surface, although it is not clear whether von Willebrand factor (VWF), in addition to fibrinogen, also plays some role in stabilizing platelet thrombi. When blood containing one of the anti-GP IIb/IIIa agents began to be perfused, ligand bound with activated GP IIb/IIIa was replaced by the anti-GP IIb/IIIa agent. Parts of the platelet thrombi started to become detached when a certain number of ligands were replaced by anti-GP IIb/IIIa and the strength of the GP IIb/IIIa ligation was no longer sufficient to support the integrity of the thrombi. Details are explained in the "Discussion" section.
and may dissociate into small aggregates incapable of embolization. Nonetheless, our experimental results might have in vivo relevance because anti-GP IIb/IIIa agents tend to be used in combination with anticoagulant and thrombolytic therapy, which dissolve fibrin net. Indeed, we have shown that the dissolution effects of anti-GP IIb/IIIa agents could still be seen when blood was anticoagulated by heparin at concentration achieved with clinical use.

Another important issue we could not completely address in this paper was the effects of time on the stability of platelet thrombi. Indeed, we could not demonstrate whether the dissolution effects we described herein still occurred when the subsequent perfusion of blood containing anti-GP IIb/IIIa agents was started hours after the formation of platelet thrombi. This issue is relevant because the binding of fibrinogen to activated GP IIb/IIIa was reported to be reversible initially and became irreversible later (35). We could not conduct experiments to answer this question directly, because continuous perfusion, which prevents the falling of leukocytes onto the platelet thrombi because of gravity, was necessary to ensure exclusion of the possible effects of leukocytes on the stability of the platelet thrombi. The dissolution effects of anti-GP IIb/IIIa agents, although not exactly demonstrated as described in this study, could be seen even when the subsequent perfusion of blood containing anti-GP IIb/IIIa agents started to be perfused after 30 min from the end of initial blood perfusion (data not shown).

**Conclusions.** We have previously shown that different anti-GP IIb/IIIa agents might have different effects on platelet activation under high shear stress condition (36,37). Although there is still ongoing debate on whether or not different anti-GP IIb/IIIa agents have different antithrombotic effects in vivo (24,38), it would be reasonable to suppose that all three anti-GP IIb/IIIa agents have similar effects on thrombus dissolution when used in doses required to block platelet aggregation. In conclusion, we have demonstrated the dissolution effects of various anti-GP IIb/IIIa agents on platelet thrombi formed on the collagen surface under blood flow conditions.

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**REFERENCES**


