A Structural and Dynamic Investigation of the Facilitating Effect of Glycoprotein IIb/IIa Inhibitors in Dissolving Platelet-Rich Clots

J.Ph. Collet, G. Montalescot, C. Lesty, J.W. Weisel

Abstract—Glycoprotein IIb/IIIa (GP IIb/IIIa) inhibitors were shown recently to facilitate the rate and the extent of pharmacological thrombolysis. However, their synergistic potential with rtPA in dissolving thrombotic vaso-occlusions is not fully understood. We have therefore developed a dynamic and structural approach for analysis of fibrinolysis to assess the inhibiting effect of platelets and the facilitating effect of GPIIb/IIa inhibitors in dissolving platelet-rich clots (PRCs). Fluorescent rtPA was used to study the architecture of PRCs, to follow the progression of the rtPA binding front, and to measure the lysis-front velocity using confocal microscopy. Fibrinolysis resistance of PRCs was related to a reduction of both rtPA binding and lysis-front velocities of platelet-rich areas compared with platelet-poor areas (2.4±0.2 versus 3.5±0.4 μm/min for rtPA binding velocity, P=0.04, and 1.2±0.6 versus 2.8±0.2 μm/min for lysis-front velocity, P=0.008, in platelet-rich and platelet-poor areas, respectively). Fibrinolysis appeared heterogeneous, leaving platelet-rich areas un-lysed. Adding pharmacological concentrations of abciximab (0.068 μmol/L) or epifibatide (1 μmol/L) before clotting decreased the average surface of platelet-rich areas by 64% (P=0.0005) and 72% (P=0.0007), respectively. The resulting equalization of rtPA binding rate and rtPA binding-front velocity between platelet-rich and platelet-poor areas led to a 3-fold increase of the lysis-front velocity in platelet-rich areas of either abciximab-PRC (P=0.006) or epifibatide-PRC (P=0.03). The overall lysis rate of treated-PRC was increased by 74% compared with control-PRC (P<0.01). These results demonstrate that fibrinolysis resistance of PRCs is related primarily to the heterogeneity in the clot structure between platelet-rich and platelet-poor areas. GP IIb/IIa inhibitors facilitate the rate and the extent of fibrinolysis by improving rtPA binding velocity and, subsequently, the lysis rate in platelet-rich areas. These findings provide new insights on the synergistic potential of GP IIb/IIa inhibitors and fibrinolytic agents. (Circ Res. 2002;90:428-434.)

Key Words: fibrin ■ platelets ■ thrombolysis ■ fibrinolysis ■ inhibitors

Platelets play a pivotal role in the fibrinolysis resistance of occluding coronary thrombi, and the benefit of lytic agents in patients with acute myocardial infarction is further improved with concomitant administration of aspirin.1 More recently, direct inhibition of platelet interactions with fibrinogen by abciximab (ReoPro) has been shown to restore coronary flow2 and to improve the outcome of acute myocardial infarction patients undergoing either primary angioplasty or thrombolysis.3–8 In particular, the ability of abciximab alone to restore coronary vessel patency in 17% to 32% of the patients has been related in vitro to its potential to disaggregate preestablished platelet-rich clots (PRCs) and to limit clot extension.9,10 However, whether these abciximab-mediated structural changes of PRCs can account for the acceleration of the fibrinolysis rate is unknown.

To evaluate the inhibitory effect of platelet aggregates on fibrinolysis and the facilitating effect of GP IIb/IIa inhibitors in dissolving PRCs, we developed a dynamic and structural approach to the study of in vitro fibrinolysis. Lysis of native, fully hydrated PRCs was initiated with fluorescently labeled recombinant tissue type plasminogen activator (FITC-rtPA). The binding rate of FITC-rtPA, the binding-front velocity or rate of movement of the FITC-rtPA binding front, and the lysis-front velocity in PRCs were carefully monitored over time by scanning laser confocal microscopy. These parameters were measured in platelet-rich and platelet-poor areas of control-PRC and of PRCs obtained by adding...
abciximab or eptifibatide prior to clotting. Our study reveals that platelet-rich areas exert a critical effect on fibrinolysis of PRCs and that the facilitating effect of GP IIb/IIIa inhibitors is related to structural changes of these areas.

Materials and Methods

Material
Abciximab (ReoPro) and eptifibatide (Integrillin) were supplied by Eli Lilly & Company and Shering-Plough as a 2 mg/mL solution and were dissolved in 0.15 mol/L NaCl, 0.01 mol/L Tris/HCl, pH 7.4. Aspirin (Aspetic, Synthalabo) was supplied as a 100 mg/mL solution. Human thrombin (Enzyme Research Laboratories, Inc) was stored as a 1000 IU/mL solution. RtPA (Actilyse) was from Boehringer Ingelheim, and fluorescein isothiocyanate (FITC) was from Sigma.

Preparation of Platelet-Rich Clots
Venous blood from healthy informed volunteers (n = 10) was anticoagulated (1 volume of 0.13 mol/L citrate for 9 volumes of blood) and centrifuged to obtain platelet-rich plasma with a final platelet count of 125 000/μL. Control-, abciximab-, and eptifibatide-PRC were obtained by clotting 0.12 mL of platelet-rich plasma in the presence of either buffer, or 0.068 μmol/L of abciximab or 1 μmol/L of eptifibatide with CaCl2 (10 mmol/L) and thrombin (0.125 IU/mL). Such unphysiologically high concentrations of Ca2+ were used to overrun the chelating effect of citrate. Aspirin was added in platelet-rich plasma before the initiation of clotting at a final concentration of 100 μg/mL to completely inhibit platelet cyclooxygenase activity.1 Abciximab and eptifibatide final concentrations (or buffer) corresponded to an intravenous bolus of 0.25 mg/kg and 4 μg/kg, respectively. These concentrations produce >80% saturation of the binding platelet surface GP IIb/IIIa receptors to inhibit thrombosis in vivo.12,13 Glass microchambers designed for microscopy experiments were filled with 0.08 mL of the different clotting solutions and clotting proceeded for 20 minutes in a moist atmosphere at 37°C (Figure 1).

Lysis Experiments
RtPA was labeled with FITC as previously described.14 Briefly, FITC (50 μg/mL) was added to a stock solution of 2 mg/mL of rtPA. After 1 hour of incubation, free FITC was removed by gel filtration. The A494/A280 ratio in preparations of FITC-rtPA was 1.6, corresponding to 2 FITC molecules per molecule of rtPA. FITC-rtPA was dissolved in platelet-poor plasma at a final concentration of 5 mmol/L and 10 μL of this solution was loaded at the edge of the PRCs and underwent a diffusion-mediated transport (Figure 1). After 15 minutes of incubation, scanning was started at regular time intervals using the regular fluorescence mode.9 Ten optical sections of 208×208 μm (512×512 pixels) were collected at intervals of 1 μm in the Z-axis and were projected and combined into 1 image, generating 2-dimensional reconstructed images of the PRCs.

Morphological Analysis
Areas of platelet aggregates within PRCs were specifically and automatically detected and quantified (S. ag in μm²) using the Visilog software (version 5.0) and the “the watershed line” transformation technique as previously described.9,10,16 The average number of fibers (number of fibers/volume) was determined either in platelet-poor or platelet-rich areas by counting the fibers in squares of 17×17 μm (42×42 pixels).

The lysis-front velocity was measured within the same scanned area of 208×208 μm in both platelet-rich and platelet-poor areas. For that purpose, 5 lines perpendicular to the edge of the lysis front were drawn and the lysis-front velocities were determined at constant time intervals according to the position of the line relative to the platelet aggregates (Figure 1).

The binding rate of FITC-rtPA, defined as the variation of optical density in a given area during a given time interval (OD/min/μm²), was determined along the same lines with the Visilog software using the same squares as defined for fiber counting (Figure 1). The binding-front velocity of FITC-rtPA, which reflects the motion of the progressive accumulation of FITC-rtPA on native fibrin in a given area, was defined as the time-scale displacement (μm/min) of the peak of the optical density of fluorescence.

Consecutive reconstructed images were also displayed in false color and merged into a single image to assess the mechanical effect of platelets on the mobility of the fibrin network during lysis. The fibers were considered immobile if their position in such images coincided.

Statistical Analysis
Statistical analysis was performed with StatView software (version 5.0, Abacus Concepts, Inc). Continuous variables were expressed as mean±SEM and differences between control and PRCs formed with abciximab or eptifibatide were determined by ANOVA. A level of α=0.05 was accepted as significant.

Results
Morphological Properties of Control and Abciximab-PRC
The architecture of PRCs labeled with FITC-rtPA looked similar to PRCs labeled with FITC.9,17 PRCs were heterogeneous in comparison to fibrin-rich clots with platelet-poor areas made of a 3-dimensional network of randomly oriented, branchiing fibers alternating with platelet-rich areas, made of bent and retracted fibers organized with some preferred orientation, with many fibers aligned radially with respect to the platelet aggregates (Figures 2A through 2C).9,10 Morphological analysis demonstrated a significantly higher number of fibrin fibers in platelet-rich areas compared with platelet-poor areas, confirming that the fibrin network is dramatically influenced by the presence of platelets (Figure 2 and Table 1). Addition of pharmacological concentrations of abciximab (0.068 μmol/L) or eptifibatide (1 μmol/L) before the initiation of
clotting decreased by 64% and 72%, respectively, the average surface area of platelet-fibrin aggregates (Figure 3 and Table 1). This led to a more homogeneous architecture of the whole fibrin network with a slight increase in the number of fibrin fibers in platelet-poor areas (Table 1).

**Lysis-Front Velocity**

The lysis front of control-PRC switched from a straight-shape to a curved-shape when adjacent to a platelet-fibrin aggregate, which was progressively completely surrounded by lysed areas (Figure 1). In some extreme cases, a finger-like pattern of lysis could be observed with persistent platelet-rich areas surrounded by completely digested platelet-poor areas. Moreover, unlike platelet-poor areas, the most obvious features were the significant reduction in the number of fibrin fibers in platelet-poor areas (Figure 1). In some extreme cases, a finger-like pattern of lysis could be observed with persistent platelet-rich areas surrounded by completely digested platelet-poor areas.

As expected, the higher fibrin concentration of platelet-rich areas was characterized by an initial latent phase of low velocity (<1.5 μm/min) followed by a high-speed phase with velocity >14 μm/min (Figure 5). Finally, after complete digestion of the platelet aggregate, the lysis front recovered a straight shape (Figure 1F).

Adding abciximab or eptifibatide prior to the initiation of clotting prevented platelet-mediated organization of the fibrin network. As a consequence, lysis speed of platelet-rich and platelet-poor areas of treated-PRC reached similar values (Table 2). The lysis front kept a straight shape with only slight deformations adjacent to the platelet aggregates, indicating a homogeneous fibrinolysis. In merged micrographs, the red band had a constant width all along the lysis front (Figure 4B). Interestingly, pharmacological concentrations of abciximab and eptifibatide displayed similar efficacy, and the overall lysis speed of treated-PRC was found to be 2 times higher than control-PRC (4.0±0.5 versus 2.3±0.3 μm/min; P<0.01).

**Binding-Rate and Binding-Front Velocity of FITC-rtPA**

As expected, the higher fibrin concentration of platelet-rich areas led to a significantly higher binding rate of FITC-rtPA in platelet-rich areas compared with platelet-poor areas (Table 2). However, after normalization to correct for the difference in fibrin density, FITC-rtPA binding rate did not differ anymore between platelet-rich and platelet-poor areas of control-PRC (0.45±0.1 versus 0.35±0.1 OD/min/fiber, P=0.7). The other major consequence of the heterogeneous structure of control-PRC was a significantly lower binding-front velocity of FITC-rtPA in platelet-rich areas compared...
with platelet-poor areas, which accounted for the differential lysis speed between platelet-rich and platelet-poor areas (Table 2).

Adding GP IIb/IIIa inhibitors prior to the initiation of clotting led to a similar binding rate and to a similar binding-front velocity for platelet-rich and platelet-poor areas, suggesting that the GP IIb/IIIa inhibitor-mediated structural changes of the PRCs directly affected rt-PA binding (Table 2). Again, similar trends were found in both abciximab and eptifibatide-treated PRC (Table 2).

### Fibrin Network Mobility and Fibrin Retraction During Fibrinolysis

Mobile fibrin fibers were visualized next to the lysis front in a narrow band of 10 to 15 μm width where green and red fibers did not overlap (Figure 4A). This mobile band was found to be 3 times wider in platelet-rich areas compared with platelet-poor areas of control-PRC (24 ± 10 versus 8 ± 2 μm; n = 12) (Figure 4A), whereas no such difference was observed in treated-PRC (Figure 4B). Fragmented fibers released from the lysis front of platelet-poor areas of control and PRCs formed with GP IIb/IIIa inhibitors tended to agglomerate as small pieces of 8 ± 3 μm (n = 12) in average diameter and of 5 minutes in average half-life (Figures 1C and 2A through 2E). In contrast, more complex agglomeration was observed in platelet-rich areas of control-PRC with agglomerates of more than 60 μm in diameter and an estimated half life up to 30 minutes. Interestingly, platelet-rich and platelet-poor areas of treated-PRC displayed similar features with fibrin debris that did not exceed 10 μm in diameter. These morphological findings further indicated that platelets can affect mechanically the shape and the mobility of the fibrin network and can interfere with fibrinolysis.

### Discussion

Although inhibition of platelet aggregation is likely to be the major mechanism of the efficacy of GP IIb/IIIa inhibitors, their proven facilitating effect in dissolving PRCs is not fully understood. Several hypotheses have been formulated. Thrombin generation initiated by tissue factor in the presence of platelets has been shown to be inhibited by abciximab and could contribute therefore to

### Table 2. Lysis Parameters in Platelet-Poor and Platelet-Rich Areas of Control- and Treated-PRC Digested With FITC-rtPA (5 nmol/L)

<table>
<thead>
<tr>
<th></th>
<th>Control-PRC (n=9)</th>
<th>Abciximab-PRC (n=5)</th>
<th>Eptifibatide-PRC (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis-front velocity within platelet-poor areas, μm/min</td>
<td>2.8 ± 0.2</td>
<td>4.1 ± 0.3*</td>
<td>4.6 ± 0.9*</td>
</tr>
<tr>
<td>Lysis-front velocity within platelet-rich areas, μm/min</td>
<td>1.2 ± 0.3</td>
<td>3.8 ± 0.4*</td>
<td>4.1 ± 0.7*</td>
</tr>
<tr>
<td>Binding-front velocity within platelet-poor areas, μm/min</td>
<td>3.5 ± 0.4</td>
<td>3.8 ± 0.5</td>
<td>4.7 ± 1.1</td>
</tr>
<tr>
<td>Binding-front velocity within platelet-rich areas, μm/min</td>
<td>2.4 ± 0.2</td>
<td>3.1 ± 0.2</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>FITC-rtPA binding rate within platelet-poor areas, OD/μm²/min</td>
<td>21.3 ± 5.0</td>
<td>18.4 ± 3.9</td>
<td>10.8 ± 1.2</td>
</tr>
<tr>
<td>FITC-rtPA binding rate within platelet-rich areas, OD/μm²/min</td>
<td>8.9 ± 3.2</td>
<td>14.5 ± 3.1</td>
<td>7.7 ± 1.7</td>
</tr>
</tbody>
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Brackets indicate a significant difference between platelet-poor and platelet-rich areas of the same clot (P<0.01).

*Significant difference (P<0.05) between PRC formed with either abciximab or eptifibatide and control-PRC.
the limitation of clot extension in vivo. In vitro, abciximab has been shown to improve fibrinolysis by increasing fibrin exposure to lytic agents. However, the precise role of platelets and GP IIb/IIIa inhibitors in the dynamic process of fibrin digestion is still lacking. The present work shows that PRC lysis resistance is related to a reduced rtPA-binding rate and to a reduced lysis-front velocity in platelet-rich areas compared with platelet-poor areas, leaving platelet-rich areas un-lysed. Adding GP IIb/IIIa inhibitors before the initiation of clotting restores a similar rtPA-binding rate and a similar lysis-front velocity between platelet-rich areas and platelet-poor areas, leading to a significant acceleration of PRC dissolution.

The results of this dynamic and structural approach demonstrate that platelet-rich areas can be effectively dissolved by fibrinolytic agents and that lysis resistance of PRC results from a significant reduction of the fibrin fiber lysis rate of platelet-rich areas compared with platelet-poor areas. This differential lysis rate accounts for the progressive deformation of the lysis front, creating the impression that fibrinolysis advances through the clots in meandering channels. Similar finger-like patterns of recanalization on a macroscopic scale have been previously described in coronary artery thrombi of animal models and in whole blood clots dissolved with lytic agents under pressure-driven permeation, but have not heretofore been seen in as much detail at a microscopic scale. Our findings suggest that these channels of recanalization are related to the fibrinolysis resistance of platelet-rich areas.

The reduction of rtPA binding-front velocity in platelet-rich areas can be explained by the high density of retracted fibrin fibers and further corroborates previous data showing that platelets decrease the binding of rtPA as a consequence of clot retraction. The reduction also accounts for the subsequent initial phase of low velocity of the lysis-front motion found in these areas. However, the high fibrin concentration of these areas yielded to a higher rate of rt-PA binding and to a complex restructuring and agglomeration of lysing fibers, which have been shown to accelerate the fibrinolysis process. This might have contributed to the late and subsequent dramatic increase of lysis-front velocity in platelet-rich areas (Figure 5). These structural findings illustrate the complex interrelationships between the mechanical platelet contractile forces and the process of thrombolysis.

Previous studies have reported that uncoupling fibrin from platelet integrin receptors using D-RGDW peptide or anti-GPIIb/IIIa monoclonal antibody can promote fibrinolysis at the platelet-fibrin interface. We have recently demonstrated that adding abciximab prior to clotting prevented platelet-mediated fibrin remodeling in a dose-dependent manner, leading to an acceleration of the overall PRC lysis rate. The present investigation provides direct dynamic and structural evidence of the consequences of these IIb/IIIa inhibitor-related changes. Platelet-rich areas recovered a similar FITC-rtPA binding rate and a similar lysis rate as platelet-poor areas, and the overall abciximab-PRC looked like a fibrin-rich clot when undergoing lysis.

Of importance is that these IIb/IIIa inhibitor-related changes were independent of the tested molecules, suggesting that the efficacy of IIb/IIIa inhibitors in dissolving PRCs is solely mediated by the inhibition of the platelet GP IIb/IIIa receptor and not to the α,β receptor. However, previous studies have reported differential efficacy between abciximab and eptifibatide in inhibiting platelet-fibrin clot retraction despite equivalent antiaggregatory potency, emphasizing that the mechanism of action of abciximab and eptifibatide are different. This further indicates that assessing the effect of...
GP Ib/IIa inhibitors in vitro depends on a great variety of parameters including the type of anticoagulants,27 the dose of GP Ib/IIa inhibitors, and the mechanical conditions of clot formation. For example, in the present study, whole blood was anticoagulated with citrate, which has been shown to overestimate eptifibatide antiplatelet effects on ADP-mediated platelet aggregation.27 To overrun the chelating effect of citrate, unphysiologically high concentrations of Ca2+ were used. In addition, clotting was obtained under static conditions using an unphysiological pathway of coagulation activation. Finally, it also emphasizes that platelet-fibrin(ogen) interactions are extremely complex and may involve receptors other than GP Ib/IIa, as recently reported with recombinant fibrinogen molecules lacking the specific GP Ib/IIa binding sites.28–29

FITC-rtPA and GP Ib/IIa inhibitors underwent a diffusion-mediated transport, resulting in a slower delivery, especially within platelet-rich areas. This experimental condition represents an important limitation of this study. It may explain why GP Ib/IIa inhibitors when added together with FITC-rtPA had no significant additional effect on fibrinolysis compared with FITC-rtPA alone, whereas we have previously reported that early pressure-driven permeation of preestablished PRCs with abciximab led to a significant improvement of fibrinolysis (~30%).9 Other important factors in addition to clot architecture could have contributed to the fibrinolysis resistance of platelet-rich areas in our experimental setting. For example, platelet activator inhibitor-1, a major determinant of the failure of thrombolytic therapy in vitro and in vivo,30–33 has been detected only in platelet-fibrin aggregates.9 Finally, whether the pharmacodynamic differences between small-molecular weight inhibitors and abiciximab can affect their efficacy as adjunctive therapy to thrombolysis in vivo remains to be established.

In conclusion, this work is the first dynamic and structural approach at the microscopic level to unravel the mechanisms of lysis resistance of PRCs. It provides new mechanistic insights to understand the efficacy of GP Ib/IIa inhibitors in combination with lytic agents in restoring early coronary patency in acute myocardial infarction.

Acknowledgments

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References


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