Blockade of Platelet GPIIb-IIIa (Integrin \(\alpha_{\text{IIb}}\beta_3\)) in Flowing Human Blood Leads to Passivation of Prothrombotic Surfaces

Markus A. Riederer, Mark H. Ginsberg¹, Beat Steiner

Department of Vascular and Metabolic Diseases, F. Hoffmann-La Roche Ltd., Basel, Switzerland, ¹Departments of Vascular Biology and Cell Biology, The Scripps Research Institute, La Jolla, California, USA

Keywords
Platelet, flow, GPIIb-IIIa, \(\alpha_{\text{IIb}}\beta_1\), trans-dominant inhibition

Summary
We examined the impact of platelet activation on platelet adhesion to collagen in flowing human blood. ADP activation of platelets in ex vivo flowing blood resulted in paradoxical inhibition of platelet deposition on collagen. Blockade of fibrinogen binding to platelets by Lamifiban, a competitive antagonist of GPIIb-IIIa (integrin \(\alpha_{\text{IIb}}\beta_3\)), reversed this inhibition, leading to a marked increase in integrin \(\alpha_{\text{IIb}}\beta_1\)-dependent platelet adhesion. Analysis of integrin \(\alpha_{\text{IIb}}\beta_3\)-dependent platelet adhesion to collagen indicated that ADP-induced suppression of platelet adhesion is the result of trans-dominant inhibition of integrin \(\alpha_{\text{IIb}}\beta_3\), caused by fibrinogen binding to integrin GPIIb-IIIa. Lamifiban blocked fibrinogen binding, reversing the trans-dominant inhibition of \(\alpha_{\text{IIb}}\beta_3\) dependent adhesion to collagen. The GPIIb-IIIa antagonist resulted in the formation of a non-thrombogenic, passivated surface comprised of an adherent platelet monolayer. This unexpected consequence of blocking fibrinogen binding to GPIIb-IIIa may explain the long-term benefits of short-term GPIIb-IIIa antagonist treatment of Acute Coronary Syndrome patients.

Introduction
Platelets circulate in a largely quiescent state until challenged by vascular injury. Agonists, which trigger platelet activation, are generated at the site of vascular damage and include ADP, serotonin, and \(\alpha\)-thrombin in the fluid phase and collagen in the extracellular matrix of the subendothelium. Platelets adhere to collagen in both a direct manner via integrin \(\alpha_{\text{IIb}}\beta_1\) (VLA-2, GPIa-IIa) and indirectly by vWF forming a bridge between collagen and platelet glycoprotein Ib (1). Following platelet adhesion and activation, platelet thrombus formation is mediated by fibrinogen binding to GPIIb-IIIa (integrin \(\alpha_{\text{IIb}}\beta_3\)) forming a bridge between platelets. Ligand binding has been reported to induce intracellular signaling and has been defined as “outside-in” signal transfer (2). This signal can regulate intracellular processes but also the function of other surface localized integrins. Such “trans-dominant integrin regulation” or “integrin cross-talk” affects cellular processes like phagocytosis (3), soluble ligand binding (4, 5) and adhesion (6, 7).

Patients with acute coronary syndrome (ACS) have increased levels of circulating activated platelets that remain activated long after clinical stabilization (8-11). Although the increased level of activated platelets are believed to mirror the extent of underlying lesions, their effects on thrombus formation are not completely understood. Here, we examined the impact of platelet activation on platelet adhesion to collagen in flowing human blood. The data presented in this report may explain the long-term benefits of short-term treatment of Acute Coronary Syndrome patients with GPIIb-IIIa antagonists (12-14).

Materials and Methods

Antibodies and Reagents
The anti-GPIIb-IIIa antibodies pl-62 (anti-GPIIb-IIIa complex), pl-79 (anti-GPIIIa), and pl-80 (anti-LIBS in GPIIb) were described previously (15, 16). FITC conjugated mAb pl-80 and antibody fragments of pl-79 were generated according to standard protocol (17). Monoclonal anti-\(\alpha_{\text{IIb}}\beta_3\) antibody R28C8 was previously described (6). Ro 44-9883 (Lamifiban) and Ro 43-5054 two GPIIb-IIIa specific non-peptide antagonists have been described previously (18). Napsagaran (Ro 46-6240) is a highly specific active site inhibitor of thrombin (19). ADP and Human collagen type III was purchased from Sigma.

Preparation of platelet-rich plasma, gel filtered platelets. After obtaining informed consent, blood was obtained by venipuncture from healthy volunteers and anticoagulated in trisodium citrate (108 \(\mu\)M final concentration). Platelet-rich plasma (PRP) was separated by centrifugation at 20°C. For 10 min at 160 x g. Gel filtered platelets (GFPs) (200-300 x 10^9 platelets/ml) were prepared by loading PRP (+ 133 nM prostaglandin I_2) on a Sepharose CL-2B column equilibrated with Ca^2+ free modified Tyrode’s buffer (133 mM NaCl, 2.7 mM KCl, 12 mM NaHCO_3, 0.4 mM NaH_2PO_4, 0.4 mM MgCl_2, 5 mM glucose, 0.2% BSA, 10 mM HEPES pH 7.4) also containing 133 nM PGL_2. After gel filtration, CaCl_2 was added to a final concentration of 1 mM and the platelets were allowed to recover for 20 min at 37°C.

Platelet Adhesion Assay
96 well plates were coated with a solution of 10 \(\mu\)g/ml human monomeric collagen type III in 0.1 M Na-Acetate pH 5.5 overnight at 4°C. The wells were washed with 1X PBS buffer and saturated for more than 30 min with 3.5% BSA in buffer A-T (150 mM NaCl, 1 mM CaCl_2, 1 mM MgCl_2 and 20 mM Tris/HCl, pH 7.4). Gel filtered platelets (GFPs) (80 \(\mu\)l) were preincubated for 5 min with various concentrations of GPIIb-IIIa antagonist and/or monoclonal antibodies. Platelets were stimulated with 10 \(\mu\)M ADP and immediately transferred into the wells. After 15 min incubation, the supernatant was gently removed and adherent platelets were washed 3X with modified Tyrode’s buffer. Adherent platelets were fixed for 30 min at room temperature with 2% paraformaldehyde in 1X PBS, washed 3X with Tyrode’s buffer and detected by the monoclonal anti-GPIIb-IIIa antibody pl-79 (1 \(\mu\)g/ml) followed by incubation with FITC conjugated mAb pl-80.
Ex vivo Perfusion Experiments

Thermanox plastic coverslips (Miles Lab, Naperville, IL) were saturated with human collagen type III (10 µg/ml) as previously described (20). Blood was drawn from the antecubital vein of a health donor and anticoagulated with the specific thrombin inhibitor Napsagatran (Ro 46-6240, 1 µM) (19). Immediately before starting the system, platelets were activated by addition of 10 µM ADP, mixed very gently and incubated for 5 min to allow fibrinogen binding. Single platelet number was determined with a Cobas Helios cell counter of blood samples taken before and after ADP activation and passage through the perfusion system, respectively. The blood was then pulled through the three parallel plate perfusion devices as previously described (20). The present study was specifically addressed at interactions that occur in laminar shear fields, therefore, no mixing device was used. Passage through the ex vivo perfusion system does not lead to significant activation of platelets or coagulation (20). Inter-individual variability in parameters such as plasma concentration of coagulation factors, platelet-, leukocyte- and erythrocyte-number introduce a inter-individual variability in adhesion in the human ex vivo perfusion system. Thus, all experiments were performed including a donor-specific control. To combine results, the observed effects are then expressed as change relative to control (% of control).

Staining of Adherent Platelets or Fibrin

Immunogold-silver staining of fibrin and platelets was performed as previously described (20). The coverslips were embedded in Merckoglass (Merck) and examined under the microscope (Zeiss Axiophot).

Quantification of Adherent Platelets by an Image Analysis System

The qualitative amount of adherent platelets per mm² was measured on an image analysis system using OPTIMAS version 4.10 software (BioScan, Edmonds, WA). The values obtained are the average of 2 determinations taken at a distance of 1 mm from the blood entrance of the coverslip.

Two-stage Perfusion Experiments

The first perfusion at venous blood flow was performed as described above except that perfusion time was increased to 10 min. To prevent contamination of Lamifiban in the second perfusion, the inlet tubing was cleaned on the outside and the perfusion system was washed for 2 min with PBS buffer. Second re-perfusion (10 min) was then performed using non-stimulated Napsagatran anticoagulated blood.

At arterial blood flow conditions, the experiments was performed following the identical procedure. However, the experiment was performed for 5 min at shear rate of 2600 s⁻¹.

Quantification of Platelet Thrombi in Semi-Thin Sections

The coverslips were embedded in Epon, and semi-thin sections, diagonally at right angle to the blood flow direction, were prepared and analyzed as described previously (20).

FACScan Analysis of LIBS Induction

Platelet rich plasma (PRP) (10 µl) in 90 µl of modified Tyrode buffer was incubated 5 min with various concentrations of Ro 44-9883 and Ro 43-5054, respectively. Then, fluorescein labeled anti-LIBS antibody pl-80 was added with 1.5 µg/ml final concentration. After 10 min incubation, the samples were diluted with 400 µl of modified Tyrode buffer and immediately analyzed. Maximal platelet associated fluorescence induced by addition of 1 µM Ro 43-5054 was defined 100%. Concentration dependent induction of LIBS is presented as % of maximal LIBS induction.

Results

Paradoxical Effects of ADP and GPIIb-IIIa Antagonist on Platelet Deposition on Collagen

In order to reproduce platelet adhesion to injured vessel wall, we passed blood anti-coagulated with Napsagatran, a specific thrombin inhibitor, through a parallel plate perfusion system at a shear rate 65 s⁻¹ and quantified platelet adhesion to immobilized human collagen type III. As expected, there was extensive platelet adhesion and thrombus formation (Fig. 1A, B). To assess the effects of a soluble platelet agonist that might be generated in an injured vessel wall, we added 10 µM ADP to the blood. Under these conditions, ADP stimulated fibrinogen binding. To our surprise, activation of platelets with ADP profoundly suppressed platelet deposition on the collagenous surface (Fig. 1A, B ADP). Suppression by ADP was not due to platelet aggregation with consequent loss of platelet delivery to the collagen surface because single platelet count was not dramatically reduced in the blood either entering or leaving the perfusion chamber (Table 1). In summary,
ADP activation of platelets paradoxically resulted in suppression of platelet deposition onto a collagenous surface.

To explore the possibility that occupancy of GPIIb-IIIa was involved in the suppression of platelet deposition onto collagen, we used the specific GPIIb-IIIa antagonist, Lamifiban. Addition of 1.0 μM Lamifiban, which is 5 times the dose required for maximal inhibition of fibrinogen binding (18), resulted in a dramatic increase in deposition of ADP-stimulated platelets (Fig. 1. ADP + Lamifiban). At conditions simulating arterial blood flow (shear rate 2600 s⁻¹), fibrinogen binding to ADP-stimulated platelets also caused a dramatic reduction of platelet adhesion to collagen (Fig. 3. B1). Platelet deposition under these conditions, was dependent on integrin α₂β₁ (Fig. 3. B1). Platelet deposition under these conditions, was dependent on integrin α₂β₁. As the binding was completely blocked by an anti-α₂β₁ antibody (R28C8) (Fig. 1. ADP + Lamifiban + anti-α₂β₁ antibody). This data confirms the dominant role of integrin α₂β₁ in platelet adhesion to collagen and thus, in this specific experimental setting, excludes a significant involvement of other collagen receptors such as GP VI or GP IV (21, 22). Furthermore, ADP mediated platelet desensitization and thus ADP-induced inactivation of the integrin α₂β₁ receptor can be excluded (23). In addition, fibrinogen binding to ADP-stimulated platelets inhibited adhesion to immobilized anti-α₂β₁ antibody to a much lesser degree than adhesion to collagen and thus confirms that integrin α₂β₁ is not internalized (data not shown).

In conclusion, ADP stimulation of platelets in flowing blood results in suppression of α₂β₁-dependent adhesion to collagen. This effect is observed at flow conditions simulating venous- and arterial-blood flow.

### Inhibition of GPIIb-IIIa Promotes Passivation of Collagenous Surfaces

Our results suggest that an increase in circulating activated platelets could decrease platelet surface coverage thus prevent passivation of pro-thrombotic surfaces. To investigate possible surface passivation, we performed two-stage perfusion experiments.

In Experiment A, perfusion with ADP stimulated blood resulted in paradoxical inhibition of platelet adhesion to the collagenous surface (Fig. 2. A1 and also Fig. 1. B, (ADP)). Subsequent “re-perfusion” with non-stimulated, Lamifiban-free blood resulted in efficient formation of platelet thrombi (Fig. 2. A2 and Fig. 2. C (Exp. A2)). Thrombus number and size was similar to control experiments exposing non-pretreated collagen surfaces (data not shown). In contrast, in experiment B, perfusion of ADP stimulated blood in the presence of Lamifiban resulted in a dense platelet monolayer (Fig. 2. B1 and also Fig. 1. B. (ADP + Lamifiban)). Subsequent, re-perfusion of this platelet monolayer with non-stimulated Lamifiban-free blood did not support thrombus growth (Fig. 2. B2 and Fig. 2. C (Exp. B2)).

In addition, at conditions of arterial blood flow (shear rate 2600 s⁻¹), two-stage perfusion experiments demonstrated, first, paradoxical inhibition of platelet adhesion (Fig. 3 A1 and B1) and second, Lamifiban-induced surface passivation of the collagenous surface (Fig. 3. B1, B2).

In conclusion, two-stage perfusion experiments at venous- and arterial-blood flow demonstrated that paradoxical inhibition of platelet adhesion prevent surface passivation of a collagenous surface. In contrast, prevention of occupancy of GPIIb-IIIa by Lamifiban results in formation of a platelet monolayer which rapidly looses its thrombogenic potential.

### Fibrinogen Binding to GPIIb-IIIa Mediates Trans-dominant Inhibition of Platelet α₂β₁-dependent Adhesion to Collagen

To further characterize this phenomenon, we examined platelet adhesion to collagen in a non-flow condition system with gel filtered platelets. In this system, platelet adhesion was completely blocked by anti-α₂β₁ antibodies and was not affected by a non-function blocking anti-GPIIb-IIIa antibody (Fig. 4). Coating with fibrillar collagen gave rise to the same results as obtained after coating of monomeric collagen. However, fibrillar collagen introduced a higher variability (data not shown) and thus experiments were continued with monomeric collagen.

Furthermore, addition of purified fibrinogen resulted in 50% reduction in platelet adhesion. This reduction in adhesion could be restored by addition of the GPIIb-IIIa antagonist Lamifiban. In addition, the Lamifiban-enhanced adhesion was completely blocked by an anti-α₂β₁ antibody (Fig. 4).

In conclusion, the experiments performed at non-flow conditions correlated very well with the data generated in slow- and fast-flow-
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Fig. 2 Inhibition of GP IIb-IIIa promotes passivation of collagenous surface at venous blood flow conditions. Two stage perfusion experiments: The photographs show cross-sectional views to the blood flow. Experiment A: First perfusion A1: Blood was activated with 10 μM ADP and perfused at venous blood flow conditions for 10 min. Re-perfusion A2: The system A1 was re-perfused for 10 min with non-stimulated blood. Experiment B: First perfusion B1: Blood was activated with 10 μM ADP in presence of 100 nM Lamifiban and perfused for 10 min. Re-perfusion B2: The system B1 was re-perfused for 10 min with non-stimulated blood. Photographs were taken with 40-fold magnification. Each photograph is a representative example of six experiments.

Panel C: Quantification of thrombus-number and thrombus-area after re-perfusion: Diagonal views at right angle to the blood flow of A2 and B2 were quantified for thrombus-number and -size per μm of the flow-width. The data represent the average of six experiments with error bars indicating standard error deviation (SED). An added asterisk indicates a statistical significant difference relative to Exp. A2 (paired T-test, P <0.01). Thrombus area/μm is indicated with reference to the right Y-axis with gray columns. Thrombus number/μm is indicated with reference to the left Y-axis with white boxes connected by a line.

Clustering of GPIIb-IIIa Is Required for Trans-dominant Inhibition of Platelet Adhesion to Collagen

Integrin clustering and induction of conformational changes, both have been reported to be induced after fibrinogen binding (24). We reasoned that such clustering could be mimicked by a dimeric anti-GPIIb-IIIa antibody pl-79 (15). Conformational changes in GPIIb-IIIa can be induced by the specific antagonist Ro 43-5054 (16). Consequently, using isolated platelets, we explored the requirements for integrin occupancy and integrin clustering in trans-dominant inhibition. A combination of saturating quantities of antibody (pl-79) and Ro 43-5054 resulted in 70% suppression of platelet adhesion to collagen. This effect required both clustering and occupancy of the GPIIb-IIIa, as addition of either the antibody or the antagonist alone failed to suppress platelet adhesion (Fig. 5A.). To explore the requirement for dimeric antibody, we compared the effects of monovalent Fab frag-
ments of pl-79 with either intact dimeric antibodies or dimeric F(ab')
2 fragments. To compensate partial loss of activity, Fab and F(ab')
2 fragments were tested up to 10-fold higher concentrations
compared to intact antibody concentrations. Both dimeric species
suppressed platelet adhesion in the presence of Ro 43-5054. In contrast,
the Fab fragments were unable to support suppression (Fig. 5B). Con-
sequently, clustering of GPIIb-IIIa is required for its capacity to sup-
press platelet adhesion to collagen.

Conformational Changes in GPIIb-IIIa are Involved
in Trans-dominant Inhibition in Platelets

Previous work has established that Lamifiban and Ro 43-5054 differ
markedly in their ability to induce conformational changes in GPIIb-
IIIa (16) and suggests that such changes may correlate with trans-domi-
nant inhibition. In the presence of saturating quantities of the antibody,
pl-79, Ro 43-5054 was a potent inhibitor of adhesion to collagen with
an IC50 = 100 nM (Fig. 6A). In sharp contrast, Lamifiban at concen-
trations as high as 30 fold above those required to completely inhibit fibrinogen binding (18) completely lacked activity. Moreover, the
capacity of these two antagonists to support trans-dominant inhibition correlated with their capacity to alter the conformation of GPIIb-IIIa as reported by pl-80, an anti LIBS antibody (Fig. 6B). Therefore, the capacity of GPIIb-IIIa antagonists to induce trans-dominant inhibition of platelet function correlates with their capacity to change the confor-
mation of integrin GPIIb-IIIa. This data further emphasizes the specific
association of trans-integrin cross-talk between GPIIb-IIIa and integrin
α5β1.
Discussion

Clinical analysis of acute coronary syndrome (ACS) patients reveals increased levels of circulating activated platelets and thus increased risk of thrombus formation (8-11). We have shown that in ex vivo flowing human blood, fibrinogen binding to ADP-activated platelets resulted in paradoxical inhibition of integrin αIIbβ3, dependent platelet adhesion to collagen. This phenomenon was observed in experimental conditions of venous and arterial blood flow. We propose that fibrinogen binding to activated circulating platelets causes decreased surface coverage and consequently impaired "passivation" of the prothrombotic surfaces. In other words, down-regulation of platelet adhesion by activated circulating platelets might result in a persistent risk of occlusive thrombotic events.

In addition, at ex vivo venous- and arterial blood flow conditions, we demonstrate that inhibition of fibrinogen binding to GPIIb-IIIa not only prevents platelet thrombus formation but also enhances the formation of a non-thrombogenic platelet monolayer. Based on this data, we suggest that GPIIb-IIIa inhibitors can promote platelet mediated surface passivation of prothrombotic surfaces formed in arterial injury e.g. after plaque rupture or angioplasty. Therefore, this mechanism might explain why short-term inhibition of GPIIb-IIIa in ACS patients for up to 72 h was shown to result in a significant reduction of clinical events up to 6 months post-treatment.(12-14).

In addition, these experiments strongly suggest that fibrinogen binding to GPIIb-IIIa can regulate the function of integrin αIIbβ3 and thereby cause a paradoxical reduction of platelet adhesion to collagen. Our data demonstrate that “trans-dominant inhibition” or “integrin crosstalk” between GPIIb-IIIa and integrin αIIbβ3 is an important control element in the regulation of platelet adhesion to collagen. Integrin crosstalk has been described as unidirectional (25). While binding of collagen to integrin αIIbβ3 and GPVI triggers activation of GPIIb-IIIa (26), our results now demonstrate that GPIIb-IIIa can also regulate the function of integrin αIIbβ3. Therefore, in human platelets GPIIb-IIIa and integrin αIIbβ3 are connected by bi-directional trans-integrin crosstalk.

This novel regulatory mechanism of platelet adhesion might expand the understanding of other recent studies. For example, perfusion of afibrinogenemic blood over a collagen surface resulted in enhanced platelet surface coverage (27). Our data suggests that, in afibrinogenemia, fibrinogen binding to GP Ib-IIIa does not occur and thus trans-dominant inhibition of integrin αIIbβ3 function is also not induced. Therefore, absence of regulation of integrin αIIbβ3 function results in enhanced platelet adhesion. Previously, it has been reported that absence of functional GPIIIa-IIb in Glanzmann’s thrombasthenia (GT) patients also resulted in an increase of adherent platelets (contact platelets) to subendothelium (28). Although, platelet adhesion to subendothelium is a complex process with multiple surface receptors involved, the GT-data is in agreement with above reports and the data presented in this manuscript. Furthermore, in another study, the axial dependence of platelet adhesion downstream of thrombus growth was profiled. Addition of an inhibitor of thrombus growth, resulted in an increase of platelet adhesion (29). Hence, platelets which flow over a growing thrombus might be activated by thrombus-released ADP and thus, fibrinogen induced suppression of integrin αIIbβ3 function reduced downstream platelet adhesion. Addition of inhibitors of fibrinogen binding prevents suppression of integrin αIIbβ3 function and thus might explain the observed increase of platelet adhesion.

In a non-flow in vitro system with washed human platelets, the degree of induced receptor clustering or conformational changes determined the extent of suppression of integrin αIIbβ3 function and thus further emphasizes the specific signaling between these two integrins. It is not clear if the trans-dominant inhibition occurs by titration of limiting signaling elements or by generation of suppressive signals. In human platelets, commercially available inhibitors of signal transduction inhibited total-platelet function before a specific effect on trans-dominant suppression could be assessed (data not shown). However, crosstalk between the same pair of integrins expressed in a CHO cell line was also not sensitive to several inhibitors of signal transduction (6) and thus suggests a signaling network constituted by a specific set of yet unknown signaling molecules.

In conclusion, the present work documents the potential significance of trans-dominant inhibition in platelets and suggests that elucidation of its mechanism could provide new therapeutic targets for prevention of thrombotic disorders.

Acknowledgements

For excellent technical assistance we thank Anita Allemann, Isabelle Guillaumat, Martine Singer, Corine Stucki, Bruno Wessner and Olivier Kuster.

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Received January 1, 2002 Accepted after resubmission July 13, 2002