Platelets

In vitro measurement of platelet glycoprotein IIb/IIIa receptor blockade by abciximab: interindividual variation and increased platelet secretion

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Background and Objectives. Inhibition of soluble fibrinogen binding to activated platelets represents the target of pharmacologic approach with antagonists of the glycoprotein IIb/IIIa (GPIIb/IIIa) complex. In this study we assessed the effects of abciximab, a recombinant chimeric Fab fraction of the antibody against GPIIb/IIIa, on several markers of platelet activation.

Design and Methods. The platelet surface expression of GPIIb/IIIa was measured by a flow cytometry technique using a two-color assay. GPIIb/IIIa was detected by FITC-conjugated antibodies in whole blood, either unstimulated or exposed to platelet stimuli. The following antibodies were used: CD41, which recognizes the IIb/IIIa complex both in activated and non-activated conformers, and PAC-1, which is directed toward the activated conformer of GPIIb/IIIa. In addition, the same blood sample was incubated with CD62 antibody to measure P-selectin, as a marker of α-granule degranulation. The effect of abciximab was also assessed by experiments carried out on shear stress-induced platelet aggregation, a test that appears to be a predictor of platelet hemostatic function.

Results. Abciximab inhibited CD41 binding to glycoprotein IIb (GPIIb) in a concentration-dependent manner and also inhibited the binding of PAC-1 to active GPIIb/IIIa. In contrast, membrane-associated P-selectin was significantly increased by the drug, which suggests that blockade of GPIIb/IIIa receptors results in an increased platelet degranulation in response to agonists. Shear stress-induced platelet aggregation was inhibited by abciximab, with a more pronounced effect on blood filtration, which represents an index of platelet aggregate formation.

Interpretation and Conclusions. Our results indicate that GPIIb/IIIa blockade by abciximab is accompanied by an increase of α-granule secretion, suggesting that different mechanisms regulate these aspects of platelet activation. The described flow cytometry technique, that allows the simultaneous *in vitro* detection of several platelet markers, is a suitable method for assessing the effects of agents which interfere with platelet function.
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Platelets are essential for hemostatic control but they are also implicated in the pathogenesis of thrombosis. The initial adhesion of platelets to thrombogenic surfaces such as damaged endothelium or ruptured atherosclerotic plaques is a crucial event that leads to platelet activation and to the formation of platelet aggregates.[1] Fibrinogen binding to the activated platelet receptor glycoprotein IIb/IIIa (GPIIb/IIIa) plays a fundamental role in the formation of thrombi as this event finally leads to homomeric platelet aggregation. The blockade of fibrinogen binding has been recently addressed as a new therapeutic approach[2-4] and antagonists of the platelet GPIIb/IIIa represent a new class of antiplatelet agents.

Abciximab (c7E3, ReoPro®), the recombinant chimeric (human/murine) Fab fraction of the antibody raised against glycoprotein IIb/IIIa has been extensively studied from the bench to the bedside.[5] Many clinical trials have assessed its antithrombotic efficacy in several clinical settings such as percutaneous transluminal coronary angioplasty (PTCA), unstable angina and non-Q-wave myocardial infarction.[6-9] The efficacy of abciximab was tested in several clinical trials[7-11] which have also highlighted the risk of major bleeding complications and established the superior safety and comparable efficacy of abciximab in combination with a low-dose, weight-adjusted, heparin regimen.[12,13]

Interindividual variation in response to abciximab has been reported both in terms of extent and duration of inhibition of platelet aggregation.[14-16] Moreover, results from one of these studies indicate that during therapy with abciximab it is possible to identify cases in which the inhibitory effect of the drug is not complete.[14]

Here we describe a simple and relatively rapid technique, based on the use of flow-cytometry, which detects the effect of abciximab on several components of platelet activation, such as the status of GPIIb/IIIa receptors and α-granule degranulation. The effect of abciximab was further investigated by experiments performed on shear stress-induced platelet aggregation.

Design and Methods

Reagents

Abciximab (c7E3F(ab)$_2$, ReoPro®) was purchased from Centocor/BV (Leiden, Netherlands). Adenosine-5-diphosphate (ADP), the stable thromboxane analog U46619 and recombinant hirudin (r-hirudin) were purchased from Sigma. The peptide TRAP (SFLLRN) which represents the first six residues of the tethered thrombin receptor ligand was from Bachem (Heidelberg, Germany). Quantum Simply Cellular® microbeads were from Flow Cytometry Standards Corp. (FCSC, San Juan PR, USA).

Antibodies

The monoclonal R-phycoerythrin (PE)- and fluorescein isothiocyanate (FITC)-conjugated antibodies CD42b and CD41, as well as isotype mouse IgG1 were from Coulter-Immunology (Hialeah, FL, USA). FITC-conjugated CD62 was from Ortho Clinical Diagnostics (Raritan, NJ, USA). The FITC-conjugated antibody PAC-1 was from Becton Dickinson, (San Josè, CA, USA). CD42b antibody is directed against an activation-independent epitope characteristic of the α-subunit of GPIb[17] and allows the selection of platelets from among other blood cells. CD41 is directed against the IIb subunit of the GPIIb/IIIa complex, thus recognizing the IIb/IIIa complex both in activated and non-activated conformers.[18] PAC-1 binds to a conformation-dependent determinant on activated GPIIb/IIIa complexes unoccupied by adhesive proteins.[19,20] CD62 recognizes P-selectin exposed on platelet membrane.

Blood collection

Blood was collected from 14 healthy donors (6 males and 8 females) by venipuncture using a 19-gauge needle and minimal stasis. Donors were between 25 and 55 years of age and had not taken any drugs for at least 15 days before blood sampling. After informed consent had been obtained, blood was drawn into plastic syringes and the first 5 mL were discarded. The blood was anticoagulated with 3.8% trisodium citrate (9:1 v/v) for the flow cytometry assays, whereas for the aggregation test the blood was anticoagulated with 500 U/mL r-hirudin. Flow cytometry and aggregation assays were performed within 1 h of blood collection. Platelet counts were performed using a Sysmex-Toa counter (Dasit, Cornaredo, Italy). Platelet markers were assessed in blood from 9 out of the 14 subjects. The concentration-response curve of abciximab was constructed for the blood from 5 subjects.

Platelet preparation for flow cytometric analysis

Whole blood was diluted with PBS to obtain 1×10^6 platelets/100 µL. Samples were exposed to abciximab or buffer for 45 min at room temperature and subsequently incubated for 15 min with agonists at room temperature. ADP, TRAP or U46619 were dissolved in saline and used at 10, 35 and 2 µM, final concentration, respectively. Last, platelets were incubated at room temperature for 20 min with saturating concentrations (10-20 µL) of the FITC- and PE-conjugated antibodies for flow cytometric analysis. The reaction was stopped with cold PBS (700 µL).

### Analysis of platelets by flow cytometry

Flow cytometric measurements were performed with a FACS Calibur flow cytometer (Becton-Dickinson), equipped with a 15 mW air-cooled 488-nm argon ion laser. The fluorescence signals of FITC and PE were detected with 530/30 nm (channel 1) and 585/42 nm band pass filters (channel 2) with correction of the spectral overlap by color compensation. The calibration of the flow cytometer was performed using standard fluorescent microbeads (CaliBRITE®, Becton Dickinson, San Josè, CA, USA). Quantum Simply Cellular, which is a mixture of four highly uniform microbead populations of the same size characterized by increasing capacities to bind mouse monoclonal IgG antibodies, was used to quantify antibody binding to platelets. The mixture includes a population of microbeads (blank) which does not bind the mouse IgG. Labeling the microbeads with a specific antibody creates a set of standards that can be used to calibrate the instrument's response to the antibody. To exclude the interference of erythrocytes and leukocytes, platelets were gated in the forward scatter versus fluorescence 2 dot-plot based on the high expression of CD42bPE. Fluorescence histograms were obtained for 10,000 CD42b positive platelets/sample recorded at a flow rate of approximately 500 cells/sec.

The FITC positive events (GPIIb/IIIa, GPIIb/IIIa in activated form or P-selectin) were determined in this population. Histograms were composed from fluorescence data obtained using a logarithmic scale of amplification. Mean FITC- and PE-fluorescence intensities were calculated from fluorescence histograms for the gated population and data analyzed by CELLQuest software (Becton-Dickinson). The results are expressed as antibody binding capacity (ABC, arbitrary units converted to a linear scale). The gate for activated platelets was set in order to include <1% of the events seen when identical platelet samples were incubated with the control murine IgG used at the same concentration as the murine monoclonal antibodies.

In experiments carried out with abciximab, increasing concentrations of the drug were incubated with whole blood for 45 min. The IC$_{50}$ for abciximab was calculated by plotting abciximab concentrations versus the percent inhibition of ABC.

Blood filtration test

Whole blood was used for the filter test. The device and technique used to perform the test have been already described.[21] Briefly, 5 mL of anticoagulated whole blood, previously incubated with abciximab or saline for 45 min, were forced at a constant pressure of 100 mmHg (room temperature) through a capillary-sized channel of polycarbonate glass-fiber (Pall U100; Pall Process Filtration, Portsmouth, UK). The glass-fibers (diameter 0.1-3.4 µm) retain particles with a diameter > 10 µm. The instrumentation (A.B.S. UK Ltd., Fareham,
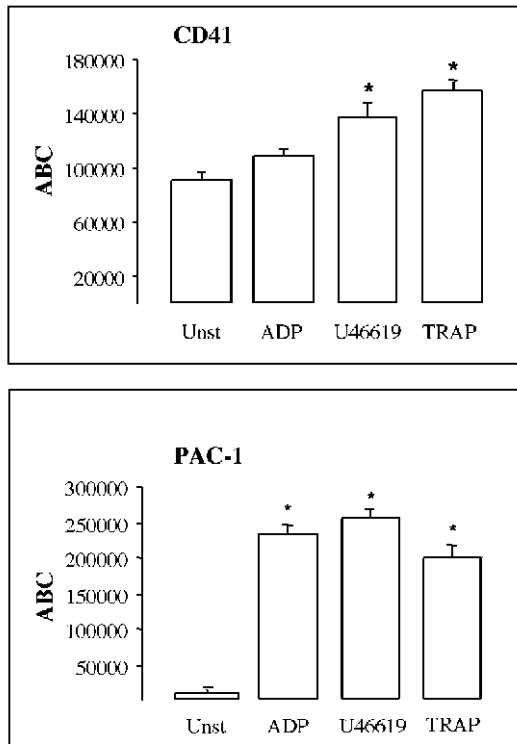
Figure 1. Effect of agonists on CD41 and PAC-1 binding to platelets in whole blood. Whole blood was diluted with PBS to obtain 1× 10^{6} platelets/100 µL and samples were incubated for 15 min with TRAP-6 (35 µM), ADP 10 (µM) and U46619 (2 µM) at room temperature. Samples were then incubated for 20 min with saturating concentrations (10-20 µL) of the FITC- and PE-conjugated antibodies for flow cytometric analysis. Incubation with isotypic control before addition of conjugated mAbs did not affect binding of antibodies. Data are expressed as antibody binding capacity (ABC) and are the mean ± SEM of 9 individual samples. *p <0.01 versus unstimulated.

Figure 2. Concentration-dependent effect of abciximab on CD41 binding to platelets. Abciximab (0.01-20 µg/mL) was incubated with whole blood for 45 min. Samples were then incubated for 20 min with saturating concentrations of the FITC-conjugated antibody CD41 for flow cytometric analysis. Data are expressed as mean ± SEM of 5 experiments performed on blood from different donors.

Hampshire, England) automatically displays the number of drops passing through the filter per time unit (5 sec). The number of platelets retained by the filter was calculated by difference between the platelet count before and after filtration in blood collected into EDTA-containing tubes. Platelet count was monitored up to 600 sec at which time blood filtration was blocked.
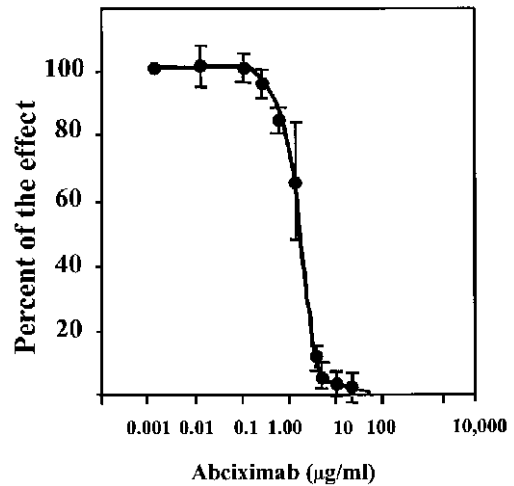
Statistical analysis

Results are presented as the mean ± SEM. Significance of differences was analyzed by ANOVA followed by Tukey's test.

Results

Effect of abciximab on GPIIb/IIIa in unstimulated platelets and platelets exposed to agonists

Platelets were identified in PBS-diluted whole blood based on the expression of the constitutive glycoprotein IIb using a PE-conjugated monoclonal antibody CD42b.

In resting platelets, GPIIb expression, recognized by CD41, was higher than that of functionally active GPIIb/IIIa conformer (not shown). The effect of ADP, TRAP-6 and U46619 on platelets was determined in whole blood exposed to each agonist for 15 min. This incubation time was selected on the basis of preliminary experiments indicating that the expression of adhesive molecules reached a plateau within 10 min after the addition of the stimulus. All the agonists used induced a characteristic activation-dependent shift to the right of the tracings of PAC-1 binding to platelets, compared with the resting condition (not shown). The same agonists, as expected, scarcely affected the constitutive expression of GPIIb/IIIa in resting platelets, measured as CD41 binding to GPIIb (not shown). The effects of agonists on the expression of both GPIIb/IIIa conformers were quantified and the results are shown in Figure 1. In preliminary experiments, abciximab was incubated with blood for 5 to 60 min. The results were that abciximab inhibited CD41 binding to GPIIb in resting platelets and that the effect was complete within 30-45 min of incubation. In this condition, an IC$_{50}$ value of 1.67 µg/mL, determined on the basis of a concentration-response curve was calculated (Figure 2). Therefore, a concentration of 2.5 µg/mL abciximab was selected and used for all the experiments. Abciximab, at this concentration, inhibited both CD41 and PAC-1 binding to platelets (Figure 3).

Effect of abciximab on P-selectin

CD62 binding to P-selectin was not affected by abciximab in resting platelets (Figure 4), whereas it increased

CD 41			
Mean ± SEM	91.7 ± 1.1	63.3 ± 3.2	70.0 ± 1.5	71.6 ± 3.0
	Unstim.	U46619	TRAP	ADP

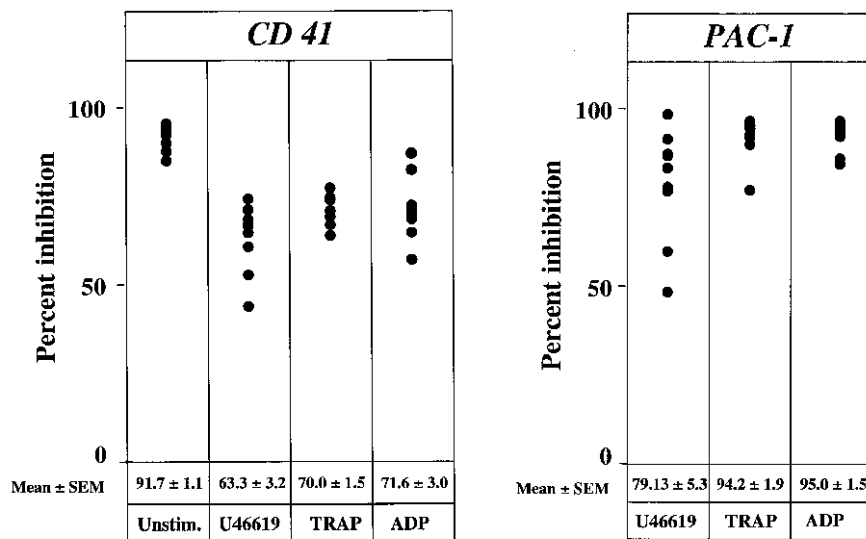| PAC-1		
Mean ± SEM	79.13 ± 5.3	94.2 ± 1.9	95.0 ± 1.5
	U46619	TRAP	ADP

Figure 3. Individual responses of samples from 9 different donors to the effect of abciximab in terms of CD41 and PAC-1 binding to platelets. Samples, incubated for 45 min with abciximab and subsequently exposed to agonists or buffer for 15 min, were treated for flow cytometric analysis as described in Figure 1. Data (mean ± SEM) are expressed as percent inhibition exerted by 2.5 μg/mL abciximab in whole blood samples from 9 donors.

significantly after blood exposure to TRAP-6 and U46619 ($1,232.5\pm197.8\%$ and $947.1\pm155.8\%$ increase over basal, respectively). The effect of ADP was less pronounced ($481.2\pm111.8\%$ increase over basal). Abciximab, pre-incubated with blood before the addition of ADP, TRAP-6 and U46619 increased P-selectin expression. The increase was statistically significant when ADP or U46619 was used as the stimulus ($p <0.05$ versus corresponding controls not exposed to abciximab) (Figure 4).

Effect of abciximab on shear stress-induced platelet aggregation

Blood filtration of untreated samples (5 mL) occurred as a function of time, with a residual blood volume of 2.23 ± 0.74 mL, n=9, measured at 600 sec. The filtration of samples treated with abciximab was complete within 83 ± 1.53 sec, n=9 (Figure 5).

Figure 5, panel a, shows an example of shear stress-induced platelet aggregation in treated and untreated blood samples from a single donor. This aggregation was measured by the number of blood drops which passed through the filter within 600 sec. Shear stress-induced aggregation was also quantified by the extent of platelet retention on the filter, evaluated at different time intervals. In untreated blood, the percentage of platelet retention was almost complete ($84.0\pm5\%$, n=9) 40 sec after the beginning of the experiment. Abciximab significantly reduced it by more than 50% (Figure 5, panel b).

Discussion

Here we describe a flow cytometric assay that allows the simultaneous evaluation of the expression of both conformers of GPIIb/IIIa and of P-selectin in whole blood. The advantages of this technique are essentially:
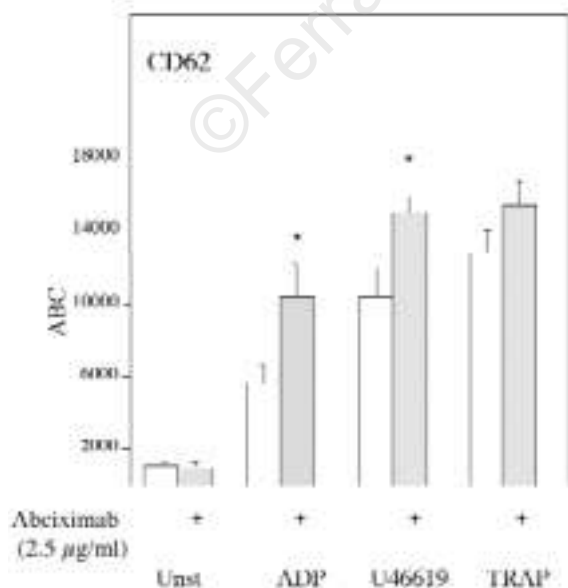
Figure 4 (left). Effect of abciximab on CD62 binding to platelets. Whole blood was incubated for 45 min with buffer or abciximab (2.5 μg/mL). Samples were then exposed to agonists (see Figure 1) and subsequently treated for flow cytometric analysis. Data are expressed as mean ± SEM, n=9, of antibody binding capacity (ABC). *$p <0.05$ versus corresponding samples not exposed to abciximab.
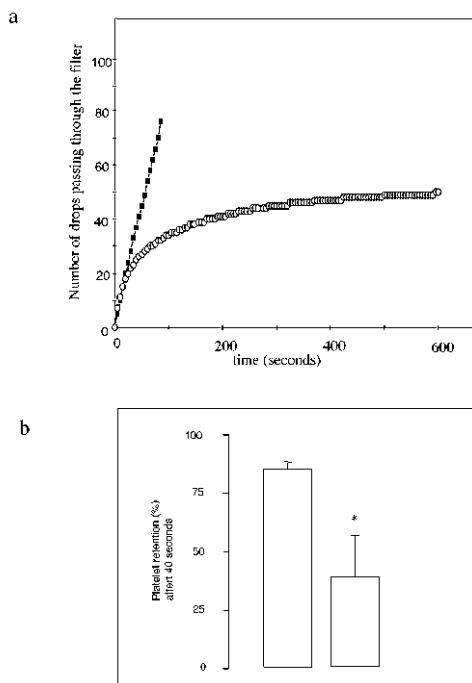
Figure 5. An example of the effect of abciximab on shear stress-induced platelet aggregation. Panel a. Platelet aggregation was measured by the number of blood drops passed through the filter within 600 s in untreated blood samples (open circles) and in samples treated with abciximab (filled squares). For details see *Design and Methods*. Panel b. Platelet retention on the filter measured 40 s after the beginning of the test, in blood samples either untreated (open column) or treated with abciximab (filled column). Data are expressed as percent of platelet retention and are the mean + SEM of 9 single experiments. *p <0.01 versus untreated samples.

a) small amount of blood needed;
b) time saving;
c) possibility of using whole blood, which greatly reduces platelet activation due to sample manipulation and avoids the loss of large platelets in the preparation processes;
d) widespread feasibility (due to the utilization of commercially available antibodies).

Our results indicate that the response of platelets from healthy volunteers to the inhibitory effect of abciximab is characterized by a degree of interindividual variability, a finding that is in accordance with that reported by others.[22] This aspect might be more relevant in a pathologic setting in which the initial levels of activated platelets are globally much greater than those seen for healthy subjects. Indeed, a marked interpatient variability in response to abciximab has also been detected during and after the drug administration to patients with coronary artery disease selected for PTCA.[14] This variability is, therefore, an important feature that must

be taken into account in view of a refinement of dosing for prolonged therapy and for the evaluation of unoccupied receptors prior to invasive procedures. From this study it emerges that abciximab markedly increased the extent of P-selectin expressed on activated platelet surface. This observation indicates that GPIIb/IIIa blockade does not inhibit, and indeed may even increase α-granule secretion, thus suggesting a discoupling between the effect of abciximab on platelet aggregation and secretion, as suggested by others.[23] It can, therefore, be concluded that different mechanisms regulate platelet granule secretion and platelet aggregation. A potentiation of ADP-induced α-granule degranulation, detected by surface expression of P-selectin, has been described by Schneider *et al.* in response to abciximab.[24] These authors postulated that fibrinogen, via a mechanism independent of its binding to GPIIb/IIIa, is responsible for this effect. On the basis of these considerations it can be envisaged that GPIIb/IIIa receptor blockade by abciximab increases fibrinogen availability, thus inducing platelet degranulation in response to agonists. The finding of increased P-selectin exposure in activated platelets following incubation with abciximab might explain the observation of increased P-selectin expression during abciximab infusion in platelets from some patients with coronary artery disease who had undergone PTCA.[14] P-selectin exposed on activated platelets mediates platelet interaction with leukocytes and therefore has a prominent role in the cross-talk between blood cells. It drives the first step (rolling) in leukocyte extravasation during the inflammatory response and the platelet adhesion to microvascular endothelium during post-ischemic reperfusion.[25,26] P-selectin increase in platelets, due to blockade of GPIIb/IIIa, can promote platelet-leukocyte or platelet-endothelial cell interaction with accumulation of pro-inflammatory chemokines and consequent amplification of ischemic damage. Abciximab, on the other hand, has been shown to reduce the interaction of platelet-leukocyte aggregates with the microvasculature and to limit inflammatory responses during reperfusion by inhibiting the surface expression of MAC-1. This effect has been observed in an animal model of myocardial reperfusion injury after coronary occlusion[27] as well as in patients with acute myocardial infarction.[28]

On the basis of these considerations it is not easy to predict the clinical relevance of P-selectin increase in response to abciximab.

In this study the effects of abciximab were confirmed by experiments carried out on shear stress-induced platelet aggregation, a test that appears to be a predictor of platelet hemostatic function.[21] Abciximab, at concentrations completely blocking platelet GPIIb/IIIa, markedly increased blood filtration, which indicates that shear stress-induced platelet aggregation is dependent on the GPIIb/IIIa glycoprotein complex. Platelet retention on the filter, was, instead, reduced only in part (by fifty percent), suggesting that aspects of blood cell interaction other than platelet aggregation might be

involved in this phenomenon. The increased availability of P-selectin on platelets exposed to abciximab might increase the extent of platelet-leukocyte aggregates, thus influencing platelet retention.

The described technique, that simultaneously detects *in vitro* the effect of abciximab on GPIIb/IIIa and on platelet α-granule secretion, represents a useful tool in order (i) to characterize the profile of platelet activation (ii) to assess the potentially paradoxical effect of agents interfering with platelet function.

Contributions and Acknowledgments

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Potential implications for clinical practice

We have assessed the effects of the GPIIb/IIIa receptor inhibitor abciximab on platelet activation by a flow cytometry technique that allows the simultaneous in vitro detection of both conformers of GPIIb/IIIa and of P-selectin. Results indicate that the extent of GPIIb/IIIa blockade is characterized by interindividual variability and results in an increase of P-selectin, indicating that platelet aggregation and secretion are regulated by different mechanisms. This technique is therefore useful to characterize the profile of platelet activation and to assess the potentially paradoxical effect of agents interfering with platelet function.

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