

Flow Cytometric Monitoring of Glycoprotein IIb/IIIa Blockade and Platelet Function in Patients With Acute Myocardial Infarction Receiving Reteplase, Abciximab, and Ticlopidine

Continuous Platelet Inhibition by the Combination of Abciximab and Ticlopidine

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Background—Improvement of thrombolysis may be achieved by concomitant strong platelet inhibition. To monitor platelet function in patients with myocardial infarction (n=46) who were treated with the fibrinolytic agent reteplase, the glycoprotein (GP) IIb/IIIa blocker abciximab, and the ADP receptor antagonist ticlopidine, we developed a flow cytometric assay.

Methods and Results—Binding of abciximab to platelets was directly monitored as the percentage of platelets stained by a goat anti-mouse antibody. Blood drawn 10 minutes and 2 hours after the start of therapy with reteplase and abciximab and during the 12-hour infusion of abciximab demonstrated a maximal blockade of GP IIb/IIIa (10 minutes, $86.2 \pm 10.3\%$; 12 hours, $85.8 \pm 7.1\%$). Starting at 24 hours, abciximab binding gradually decreased (24 hours, $74.6 \pm 16.2\%$; 48 hours, $66.8 \pm 14.9\%$; 72 hours, $60.5 \pm 16.7\%$; 96 hours, $49.4 \pm 17.8\%$; 120 hours, $35.8 \pm 16.4\%$; and 144 hours, $29.9 \pm 15.3\%$). Binding of a chicken anti-fibrinogen antibody to platelets, indicating the level of functional blockade of GP IIb/IIIa, was inversely correlated with the binding of abciximab ($r = -0.72$, $P < 0.0001$). In blood drawn at 10 minutes, platelet aggregation was maximally inhibited but recovered within 48 hours even if the majority of GP IIb/IIIa receptors were still blocked by abciximab. Reteplase did not influence abciximab binding and did not activate platelets, as measured by P-selectin expression, fibrinogen binding, and platelet aggregation. Platelet inhibition that was achieved during the first 24 hours by abciximab was directly maintained by additional treatment with ticlopidine.

Conclusions—Flow cytometric monitoring of platelet function allows differentiation of the effects of reteplase, abciximab, and ticlopidine. The combination of abciximab and ticlopidine is an attractive therapeutic strategy that provides a fast and continuous platelet inhibition. (*Circulation*. 2000;102:1490-1496.)

Key Words: platelets ■ thrombolysis ■ inhibitors

Limited success in the restoration of coronary blood flow and in the reduction of reocclusion in thrombolysis has been attributed to platelet activation.¹⁻⁴ Maximal platelet inhibition can be achieved by the blockade of the platelet fibrinogen receptor glycoprotein (GP) IIb/IIIa.^{5,6} In early experimental studies in animals, the combination of fibrinolysis and GP IIb/IIIa blockade resulted in the enhancement of thrombolysis and reduced reocclusion after recanalization of coronary arteries.^{1, 7-9} Only very recently have the clinical benefits of this combinatorial therapy been demonstrated in

clinical trials.¹⁰⁻¹² Within one of these trials, the Global Utilization of Strategies for Total Occlusion (GUSTO)-IV pilot trial (Strategies for Patency Enhancement in the Emergency Department [SPEED]),¹¹ we evaluated platelet function of patients with acute myocardial infarction who received various combinations of 3 drugs: the fibrinolytic agent reteplase, the GP IIb/IIIa blocker abciximab, and the ADP receptor antagonist ticlopidine. For the measurement of platelet function during this complex combinatorial drug therapy, we developed a monitoring strategy that is based on

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flow cytometry. Thereby, we could address the following issues: (1) the pharmacodynamic profile of the GP IIb/IIIa blockade by abciximab in patients with acute myocardial infarction and potential influences by the comedication with reteplase and ticlopidine, (2) the influence of reteplase on platelet function, and (3) the time course of platelet inhibition by the combination of abciximab and ticlopidine.

Methods

Antibodies and Reagents for Flow Cytometric Monitoring

Abciximab binding to platelets was monitored by a dichlorotriacetyl-aminomethyl fluorescein (DTAF)-labeled polyclonal goat anti-mouse IgG+IgM (H+L) antibody (Jackson Immuno Research Laboratory). Platelet-bound fibrinogen was detected by a polyclonal fluorescein isothiocyanate (FITC)-labeled chicken antibody to human fibrinogen (Biopool) as described in detail.¹³ The anti-P-selectin monoclonal antibody was obtained from Becton Dickinson. ADP (Sigma Chemical Co) was used at a final concentration of 10 $\mu\text{mol/L}$. Modified Tyrode's buffer (50 mmol/L NaCl, 2.5 mmol/L KCl, 1.2 mmol/L NaHCO_3 , 2 mmol/L CaCl_2 , 2 mmol/L MgCl_2 , 1 mg/mL BSA, and 1 mg/mL glucose) was used for predilution of the whole blood samples.

Blood Sampling

Blood was collected by venipuncture with a 21-gauge butterfly needle from an antecubital vein. Five milliliters of blood was drawn and discarded before blood samples were drawn for analysis in a second syringe and anticoagulated with 0.106 mol/L citrate. Samples were processed within 15 minutes.

Flow Cytometry

Samples for flow cytometric analysis were prepared by a whole blood assay. Twenty microliters of whole blood anticoagulated with citrate was added to 980 μL of modified Tyrode's buffer. Fifty microliters was then added to 10 μL of appropriate antibody and agonist solution. After the samples were gently mixed, they were incubated at room temperature for 20 minutes and then diluted with 500 μL of 1 \times CellFix (Becton Dickinson) to achieve a stable fixation and to avoid further activation. Fixation after incubation with antibodies was chosen because fixation before the incubation resulted in a decrease of fluorescence staining. Flow cytometry was performed on a FACScan cytometer (Becton Dickinson) within 2 hours after the fixation step. The instrument was aligned daily by using CaliBrite beads (Becton Dickinson). Blood cells were examined with Lysis II software (Becton Dickinson), and platelets were identified by light-scatter characteristics. A gate was set around the platelet population, and platelet identity was confirmed by using the anti-GP IIb antibody SZ22. A total of 10 000 platelets were analyzed within this gate. When 10 blood samples in 5 replicates each were assayed, the coefficient of variation was 10.4%.

Platelet Aggregation

The measurement of ex vivo platelet aggregation was performed with platelet-rich plasma by the turbidimetric method in a 4-channel aggregometer (PAP-4, Biodata Corp). Platelet-rich plasma was obtained as a supernatant after centrifugation of citrated blood at 160g for 10 minutes. Platelet-poor plasma was obtained by a second centrifugation step at 2500g for 10 minutes. For aggregation, the platelet count was adjusted with platelet-poor plasma to 250/nL. Light transmission was adjusted to 0% for platelet-rich plasma and to 100% for platelet-poor plasma for each measurement. Platelet aggregation was determined as maximal change of light transmission after the addition of ADP.

Study Population and Enrollment Criteria for Patients With Myocardial Infarction

Patients (n=46) were enrolled from October 1997 until November 1998 at the intensive care unit of the Department of Cardiology, University of Heidelberg, Germany, within a substudy of the SPEED trial (a multicenter, open-labeled, parallel group trial). The enrolled patients were randomized to receive either weight-adjusted abciximab (ReoPro, Centocor) with a bolus of 0.25 mg/kg body wt, followed by a 12-hour infusion of 0.125 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ to a maximum (at 80 kg body weight) of 10 $\mu\text{g}/\text{min}$, or 2 \times 10 U reteplase (Boehringer-Mannheim) or the combination of weight-adjusted abciximab combined with reduced doses of reteplase (5, 7.5, and 10 U, single bolus each; 5 plus 2.5 U or 2 \times 5 U, 2 injections 30 minutes apart). Patients admitted within 6 hours of onset of chest pain that lasted at least 30 minutes and with significant ST-segment elevation in 2 contiguous ECG leads were randomized to one of the treatment arms. Before administration of the study medication, all patients received aspirin (500 mg IV and continuing with 100 mg given orally, starting the second day) and heparin (low dose with 60 IU/kg, \leq 4000 IU IV bolus). Further heparin administration was titrated by the activated clotting time. With one exclusion, coronary angiography was performed in all patients between 60 and 90 minutes after the start of study medication. If stent placement was necessary, patients further received 2 \times 250 mg ticlopidine per day starting with the first dose immediately after placement and lasting for 3 weeks. None of the patients developed reocclusion. One patient who developed thrombocytopenia is reported separately.¹⁴ Patency data of the SPEED trial are reported elsewhere.¹¹ The study protocol was approved by the ethics committee of the University of Heidelberg, and written informed consent was obtained from each patient.

Statistical Analysis

Outcome measures over time were analyzed by repeated-measures ANOVA. If overall intergroup differences were significant ($P < 0.05$), relevant differences at specific time points were tested by the Mann-Whitney rank sum test. To test for the strength of association, the Pearson product moment correlation coefficient was determined.

Results

Direct Monitoring of Abciximab Binding to Platelets by Flow Cytometry

Abciximab binding to platelets was monitored by flow cytometry with use of a goat anti-mouse antibody. Typical histograms are given in Figure 1. The extent of abciximab binding is measured as the percentage of platelets within a gate (M1) that allows for an unspecific binding of the goat anti-mouse antibody of \leq 1.3% of platelets in all patients (see pretreatment, Figure 1). The linearity of the flow cytometric detection of abciximab binding is shown in a representative example (Figure 2A). The concentration at which maximal binding of abciximab to platelets is achieved varies between individuals. However, saturation of abciximab binding is reached with all individuals tested at 3 $\mu\text{g}/\text{mL}$ (Figure 2A). This in vitro saturation characteristic is in agreement with a blockade of \geq 80% of the platelet GP IIb/IIIa receptors at a concentration of \approx 3.5 $\mu\text{g}/\text{mL}$, which is reported to be reached in vivo with the dosage regimen applied in the SPEED trial.¹⁵⁻²⁰

Beginning with the blood samples that were drawn 10 minutes after abciximab bolus and during the 12-hour infusion, a maximal blockade of GP IIb/IIIa (pretreatment, 0.6 \pm 0.6%; 10 minutes, 86.2 \pm 10.3%; 2 hours, 84.7 \pm 7.9%; and 12 hours, 85.8 \pm 7.1%) was achieved. Starting at 24 hours,

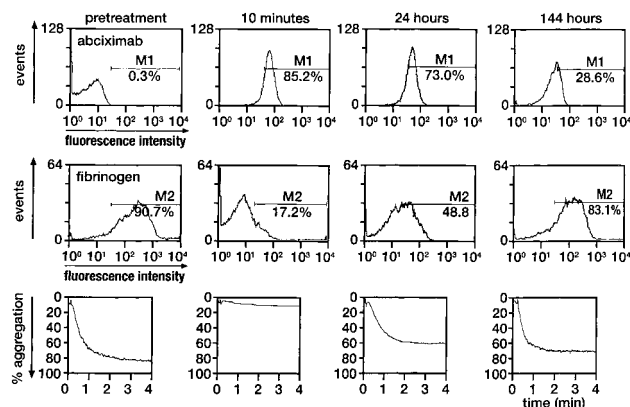


Figure 1. Representative flow cytometry histograms of abciximab and fibrinogen binding to platelets and platelet aggregometry tracings of patients treated with reteplase and abciximab. Examples are given for different time points of blood drawing (pretreatment, 10 minutes, 24 hours, and 144 hours after start of treatment). Abciximab binding was detected by using DTAF-labeled goat anti-mouse antibody. Percentage of platelets demonstrating fluorescence intensity within gate M1 were determined in each patient at each time point. Gate M1 was defined to adjust for background binding as depicted in pretreatment histogram. Fibrinogen binding to ADP-stimulated platelets was detected by using FITC-labeled chicken anti-human fibrinogen antibody. Percentage of platelets demonstrating fluorescence intensity within gate M2 is given. Gate M2 was defined to adjust for background binding of fibrinogen to nonstimulated platelets. Aggregometry was performed after addition of ADP to platelet-rich plasma and quantified as change of light transmission.

abciximab binding gradually decreased (24 hours, $74.6 \pm 16.2\%$; 48 hours, $66.8 \pm 14.9\%$; 72 hours, $60.5 \pm 16.7\%$; 96 hours, $49.4 \pm 17.8\%$; 120 hours, $35.8 \pm 16.4\%$; and 144 hours, $29.9 \pm 15.3\%$). Representative histograms are shown in Figure 1. All histograms revealed a unimodal pattern of abciximab binding to platelets. Unimodal patterns of platelet staining were also present when samples were fixed immediately after blood drawing (also at the 10-minute time point of blood drawing), arguing against a significant redistribution of abciximab on platelets during the *in vitro* incubation and preparation steps.

Inhibition of Ligand Binding to GP IIb/IIIa by Abciximab

To evaluate the functional effect of the blockade of GP IIb/IIIa by abciximab, binding of fibrinogen and the monoclonal antibody PAC-1 (specific for activated GP IIb/IIIa) was monitored. The binding of an FITC-conjugated chicken polyclonal anti-fibrinogen antibody was determined as percentage of ADP-stimulated platelets within the gate M2 (see Figure 1). *In vitro* incubation of platelets with various concentrations of abciximab revealed saturation with maximal inhibition of fibrinogen binding reached between 2 and 3 $\mu\text{g/mL}$. Abciximab binding and inhibition of fibrinogen binding to platelets are inversely correlated with each other ($r = -0.72$, $P < 0.0001$). In blood of patients that was drawn 10 minutes after the abciximab bolus, fibrinogen binding to platelets already reached minimal binding levels that remained stable at 2 and 12 hours (Figure 1 and 3B). Beginning at 24 hours, fibrinogen binding to platelets gradually recovered (Figure 1 and 3B). As for the binding of fibrinogen to

platelets, similar results were obtained with the activation-specific FITC-conjugated mouse IgM monoclonal antibody PAC-1 (data not shown).

Effects of Abciximab on Platelet Aggregation

A widely used functional evaluation of platelets is ADP-induced platelet aggregometry. *In vitro* evaluation of various abciximab concentrations revealed saturation of the inhibitory effect of abciximab at 2 $\mu\text{g/mL}$ in platelet aggregometry (Figure 2C). Ten minutes after administration of the abciximab bolus, platelet aggregation was reduced to a minimal level in the blood of patients (Figure 1 and 3C). Measurements at later time points revealed a continuous recovery of platelet aggregation. In the patient group without ticlopidine, platelet aggregation returned to normal as early as 48 hours after the start of treatment. At this time point, 70% of platelets demonstrated fluorescence intensity within gate M1 (Figure 3A). Thus, ADP-induced platelet aggregation demonstrated recovery even if the majority of GP IIb/IIIa receptors on the platelets were still occupied and blocked by abciximab.

Effects of Reteplase on Abciximab Binding and on Platelet Function

Reteplase did not influence the time course of abciximab binding to platelets as demonstrated in the comparison of the patients with and without fibrinolytic therapy (Figure 4A). Effects of reteplase on the platelet activation status can be measured by the expression of P-selectin on the platelet surface.²¹ The comparison of patients treated with reteplase only, abciximab only, and the combination of reteplase and abciximab did not reveal a significant influence of these drugs, either alone or in combination, on the platelet activation status (Figure 4B). Furthermore, reteplase did not affect the functional blockade of GP IIb/IIIa, which is a consequence of the binding of abciximab. No statistically significant difference was obtained in the comparison of fibrinogen binding to platelets in patients treated with abciximab alone or the combination of abciximab and reteplase (Figure 4C). In addition, application of reteplase alone did not cause an increase in fibrinogen binding to GP IIb/IIIa (Figure 4C). Platelet aggregation was inhibited by abciximab independent of the comedication with reteplase (Figure 4D). Treatment of patients with reteplase alone did not result in an increased platelet aggregation (Figure 4D).

Effects of Ticlopidine

Patients who required stent placement were additionally treated with ticlopidine. The gradual loss of abciximab binding to platelets did not differ between the patients treated with or without ticlopidine (Figure 3A). However, recovery of the binding of fibrinogen and PAC-1 (not shown) and platelet aggregation differed significantly in both groups (Figure 3B and 3C). In ticlopidine-treated patients, platelet inhibition reached a plateau 24 hours after the onset of treatment that was maintained thereafter, independent of further decrease in abciximab binding.

Discussion

Monitoring of Abciximab Binding to Platelets

Despite the widespread use of GP IIb/IIIa blockers, limited pharmacodynamic data on this new group of antiplatelet

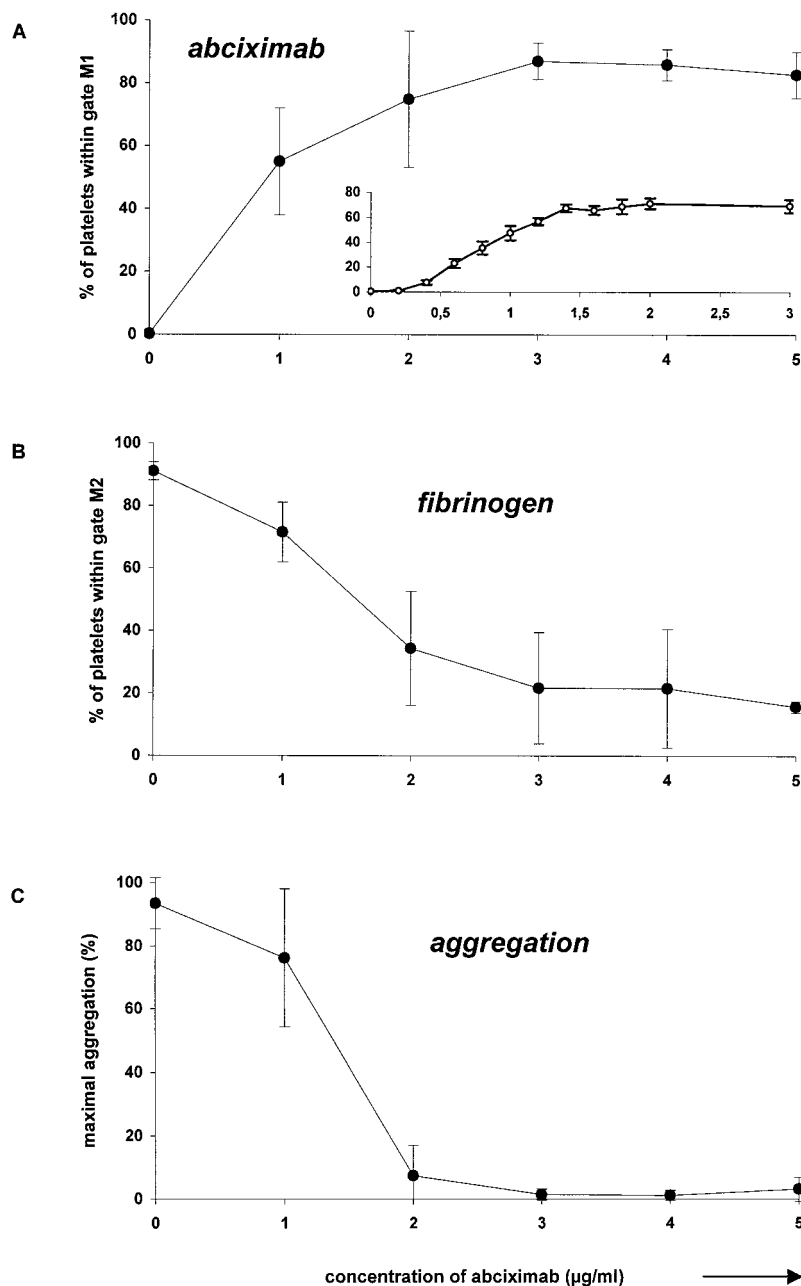


Figure 2. Binding of abciximab and fibrinogen to platelets as determined by flow cytometry and platelet aggregation at various concentrations of abciximab in vitro. A, Abciximab binding was detected by using DTAF-labeled goat anti-mouse antibody. ● indicates mean±SD of binding of abciximab to platelets of 7 individuals; ○, typical binding curve in one individual depicted as mean±SD of 5 determinations each. B, Fibrinogen binding to ADP-stimulated platelets was detected by using FITC-labeled chicken anti-human fibrinogen antibody. C, Platelet aggregation was initiated by the addition of ADP to platelet-rich plasma and quantified as maximal change of light transmission. Mean±SD values are given for 7 individuals in panels B and C.

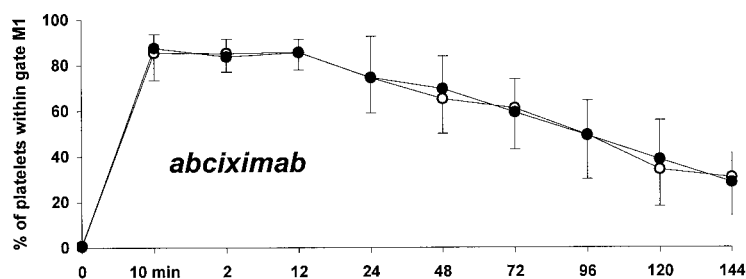
agents is available.^{16,20} Furthermore, the few strategies to monitor platelet function that have been described so far are discussed controversially.²² In the present study, a direct flow cytometric monitoring of the binding of the GP IIb/IIIa blocker abciximab to platelets is reported. In contrast to radiometric measurements that only allow the determination of the average binding of abciximab to all platelets, flow cytometry allows direct monitoring of abciximab binding to the individual platelet. This methodological advantage of flow cytometry is the basis for an important conclusion drawn from the presented data. At each time point evaluated during the present study, abciximab was evenly distributed to all platelets, as demonstrated in a unimodal fluorescence pattern. Because newly generated platelets must have entered the circulation during our 7-day monitoring period, there had to

be a continuous redistribution and, thus, equilibration of the number of abciximab molecules bound to the individual circulating platelet. The pharmacodynamic profile of abciximab binding was identical in patients receiving abciximab in combination with reteplase or abciximab alone with or without the additional administration of ticlopidine. In healthy volunteers¹⁹ and in patients with stable angina,¹⁶ similar profiles of abciximab binding to platelets have been obtained.

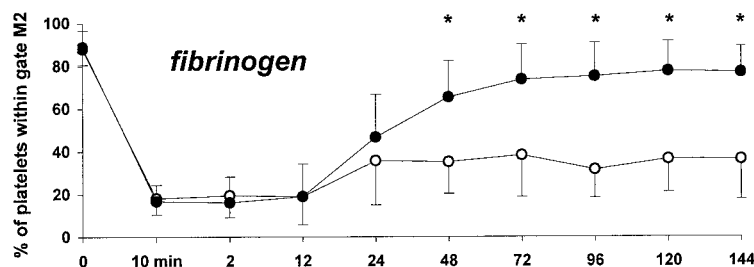
Monitoring of GP IIb/IIIa Blockers: Flow Cytometry Versus Aggregometry

Flow cytometry allows the direct measurement of the binding of abciximab and the direct determination of the extent of blockade of the GP IIb/IIIa receptors on the individual

A



B



C

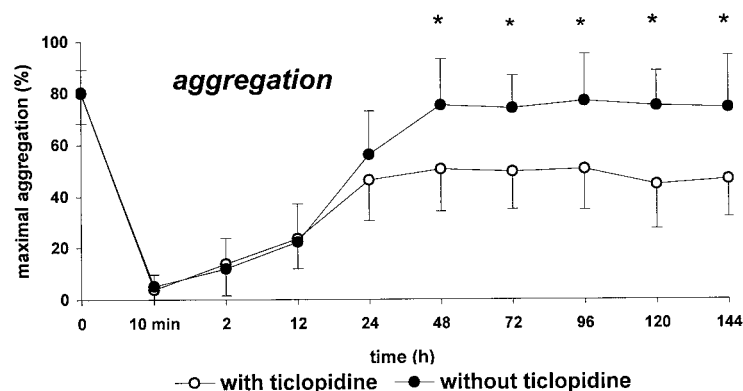


Figure 3. Time course of abciximab and fibrinogen binding and of platelet aggregation during treatment with reteplase, abciximab, and ticlopidine. Results are separately depicted for patients either receiving ticlopidine ($n=25$) or not ($n=14$). A, Percentage of platelets demonstrating fluorescence intensity of goat anti-mouse antibody within gate M1 at indicated time points. B, Monitoring of percentage of ADP-stimulated platelets demonstrating fluorescence intensity of chicken anti-human fibrinogen antibody within gate M2. C, Monitoring of maximal aggregation of ADP-stimulated platelets as determined by change of light transmission. Mean \pm SD values are given. * $P<0.001$ for intergroup comparisons.

platelet. Furthermore, the activation state of the individual platelet can be evaluated by activation-specific epitopes.²¹ In contrast, platelet aggregometry determines the overall aggregatory function of the mixture of all platelets in the sample. Platelet aggregometry has some practical limitations. The necessary centrifugation of blood samples may activate platelets. Whereas only 1 μL blood per sample is needed in flow cytometry, $\approx 500 \mu\text{L}$ blood per sample is needed for standard aggregometry. Platelet aggregation is dependent on plasma factors, especially fibrinogen. Thus, in patients treated with fibrinolytics, which result in the degradation of fibrinogen, platelet aggregation may be influenced without change in platelet function. Furthermore, platelet aggregometry cannot be used in patients with severe thrombocytopenia. In contrast, flow cytometry allows monitoring of platelet function at low platelet counts and has even been useful in the evaluation of mechanisms of GP IIb/IIIa blocker-induced thrombocytopenia.¹⁴ In platelet aggregometry, the sensitivity for the detection of partial GP IIb/IIIa blockade is limited and depends on

the stimulus used.¹⁹ Our data demonstrated a return to normal platelet aggregation even with the majority of GP IIb/IIIa receptors being blocked. Overall, flow cytometry and platelet aggregometry are 2 methods, both with specific limitations, describing platelet function on different levels, but both techniques are a useful combination in the monitoring of platelet function during GP IIb/IIIa blocker therapy.

Platelet Function in Relation to GP IIb/IIIa Blockade

Within 48 hours, ADP-induced platelet aggregation returned to normal. This early recovery despite the blockade of the majority of GP IIb/IIIa receptors on circulating platelets is consistent with pharmacodynamic data published until now.^{16,19,20,23} However, there is a caveat in the interpretation of these findings. Platelets may still be functionally inhibited; thus, platelet aggregation may be induced by weak physiological stimuli, and the stability of aggregates and platelet adhesion may be impaired even if only a low percentage of GP IIb/IIIa receptors are blocked by abciximab.

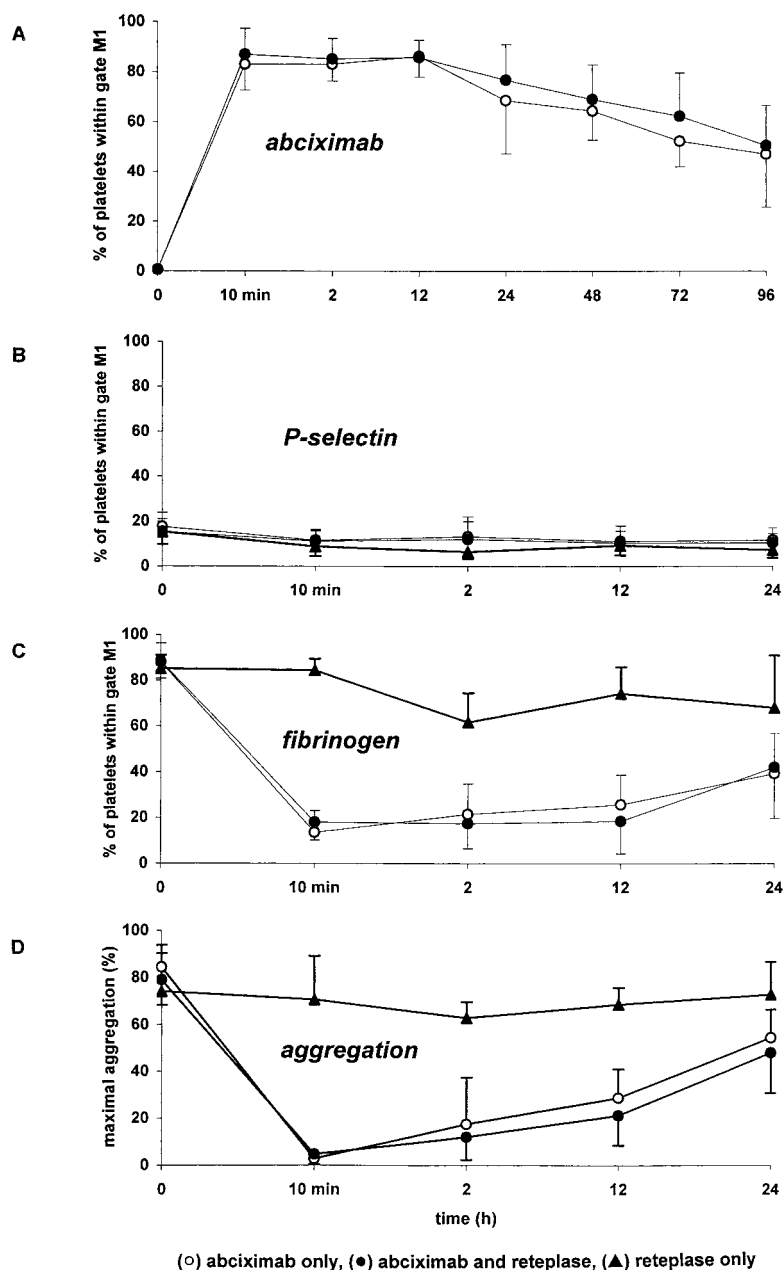


Figure 4. Time course of abciximab binding and platelet function in patients treated with abciximab, reteplase, and the combination of both. ○ indicates abciximab only (n=7); ●, abciximab and reteplase (n=32); ▲, reteplase only (n=7). A, Abciximab binding to platelets (goat anti-mouse antibody). B, Platelet activation (anti-P-selectin antibody). C, Fibrinogen binding (chicken anti-human fibrinogen antibody). D, Platelet aggregation (ADP-induced). Mean±SD values are given. No significant differences were obtained between patients treated with abciximab and reteplase and patients treated with abciximab alone (ANOVA for repeated measures).

Clinical Implications

Few data are available on the lack of time between the start of ticlopidine medication and the onset of platelet inhibition. Time periods between 2 and 5 days are reported.²⁴ The present study adds the finding that the platelet inhibitory effect of ticlopidine is complete within 48 hours. Furthermore, we describe a continuous platelet inhibition that starts after application of the abciximab bolus, is maintained throughout the abciximab infusion for 12 hours, and finally continues on a plateau level of platelet inhibition caused by the daily administration of ticlopidine. The continuity of this platelet inhibition makes the combination of abciximab-mediated GP IIb/IIIa blockade and ticlopidine very attractive.

A potential activation of platelets by fibrinolytic agents in general but especially by reteplase has been discussed.²⁵ P-selectin expression on the platelet surface is a measure of

the platelet activation status. It was not increased on the platelets of patients treated with reteplase. Furthermore, patients treated with reteplase alone did not demonstrate an increase in fibrinogen binding to GP IIb/IIIa, which also reflects the platelet activation status.²¹ And finally, platelet aggregability was not increased by the treatment with reteplase. Therefore, none of the parameters examined reported platelet activation by reteplase.

Limitations of the Flow Cytometric Monitoring of Platelet Function

Preparation of blood samples for flow cytometry, data acquisition, and data analysis requires time (15 to 30 minutes). Furthermore, trained personnel and an expensive flow cytometer are necessary. Therefore, flow cytometry is not suitable for a rapid bedside monitoring of platelet function. A recently

described rapid platelet-function assay²⁶ based on coagglutination of fibrinogen-coated beads and platelets may be one of the potential assays that may provide a fast evaluation of GP IIb/IIIa blocker effects directly in the catheter laboratory. However, for dosing decisions outside the acute situation, eg, for the monitoring of oral GP IIb/IIIa blocker therapy, flow cytometry demonstrates the advantage of the direct evaluation of the blockade of the GP IIb/IIIa receptors on the individual platelet. Because of their versatile applications, flow cytometers are available in many clinical laboratories, and the determination of the extent of GP IIb/IIIa blockade could be established as a regular laboratory test. During the preparation steps in flow cytometry and also in platelet aggregometry, GP IIb/IIIa blockers may dissociate or redistribute, and the in vitro measurements may not reflect the in vivo situation. Therefore, especially at early time points after drug administration (eg, 10 minutes) and with GP IIb/IIIa blockers that have a low affinity to GP IIb/IIIa, short incubation times or immediate fixation of the blood samples may be necessary.

Conclusions

We describe a strategy for the direct monitoring of platelet function by flow cytometry and demonstrate its use in patients with acute myocardial infarction treated by various combinations of reteplase, abciximab, and ticlopidine. The pharmacodynamics of abciximab binding in these patients could be defined, and it was not influenced by reteplase or ticlopidine. Administration of reteplase did not result in platelet activation. The administration of ticlopidine results in a continuous platelet inhibition with a direct transition of the inhibition caused by abciximab bolus and then, after infusion, to a plateau of platelet inhibition caused by ticlopidine.

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