Platelet CD62 expression and $PDGF_{AB}$ secretion in patients undergoing PTCA and treatment with abciximab

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Aims To investigate a correlation of the platelet activation marker CD62 and secretion of the growth factor PDGF from platelets in coronary patients under therapy with the GPIIb/IIIa-inhibitor abciximab.

Methods Flow cytometric assessment of fibrinogen binding (GPIIb/IIIa-binding site) and CD62 expression, as well as PDGF release of human platelets (immunoassay) and platelet aggregation with 20 μM ADP and 2 $\mu g\ ml^{-1}$ collagen were evaluated in nine patients with stable coronary artery disease. Patients were undergoing elective balloon angioplasty and were treated with aspirin (100 mg day^{-1}), heparin (ACT $<$ 220 s) and abciximab (bolus and infusion over 12 h). Blood samples were obtained before initiation of abciximab therapy (under aspirin and heparin) (I), 3 h after angioplasty under abciximab (II) and 12 h after termination of abciximab infusion (III).

Results Compared with sample I before abciximab therapy, fibrinogen binding was reduced to 37% ($\pm$34 s.d., $P<0.05$) (II) and 55% ($\pm$40 s.d., $P<0.05$) (III). Reduced fibrinogen binding also led to a significant reduction of the aggregation response to ADP (down to 37%$\pm$20) and collagen (down to 0%). Mean fluorescence intensity of CD62-expression was 78 units ($\pm$20 s.d.) (I), 72 units ($\pm$14 s.d.) (II) and 64 units ($\pm$12 s.d., $P<0.05$) (III). PDGF release from isolated, washed platelets was 99 ($\pm$33 s.d.) ng/10^9 platelets at (I), 82 ($\pm$31 s.d.) ng/10^9 platelets and 96 ($\pm$30 s.d.) ng/10^9 platelets.

Conclusions The results indicate that despite a strong reduction of GPIIb/IIIa-binding and platelet aggregation, CD62 as a marker of platelet secretion and the secretion product PDGF were only slightly reduced under abciximab treatment. No direct correlation between CD62 expression and PDGF release could be demonstrated.

Keywords: abciximab, P-selectin, platelet derived growth factor

Introduction

Glycoprotein IIb/IIIa (GPIIb/IIIa)-antagonists like the monoclonal antibody abciximab as well as RGD-peptides (integrelin) or peptidomimetics (tirofiban) have been established in prevention of reocclusion or thrombotic events in percutaneus coronary angioplasty (PTCA) and in unstable angina [1]. These compounds are strong inhibitors of platelet aggregation mediated by cross linking of fibrinogen at GPIIb/IIIa-receptors. However, late reocclusion after PTCA or in-stent restenosis are not influenced after therapy with GPIIb/IIIa-inhibitors [2]. These events are promoted by growth factors for vascular smooth muscle cells (VSMC) like platelet-derived growth factor (PDGF) or tissue-growth factor-β (TGF-β), which are released from α-granules of activated platelets [3]. Expression of CD62 (P-selectin) at the platelet surface is often used as a general marker of platelet activation [4]. More specifically, CD62 is a marker of degranulation of α-granules and acts also as ligand for platelet – leucocyte interactions, which might trigger inflammatory responses at vascular injury sites [5]. It is known that CD62 expression is enhanced in patients undergoing PTCA [6, 7], and the rate of CD62-positive platelets is even

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increased under abciximab therapy [8]. It has already been observed *in vitro* in platelets activated by ADP or thrombin-receptor activating peptide (TRAP), that GPIIb/IIIa-antagonists – despite strong inhibition of platelet aggregation – only have a minor effect on platelet granular secretion, e.g. of ATP or thromboxane A_2 [9–11]. The influence of GPIIb/IIIa-inhibitors on release of VSMC mitogens like PDGF has not been reported yet. It might furthermore be interesting, whether CD62 expression as a surrogate for platelet degranulation is in coherence with the release of platelet derived products including the active mitogen PDGF.

In this study we investigated platelets obtained from patients undergoing angioplasty and treatment with the GPIIb/IIIa-antagonist abciximab. The aim was the quantification of CD62-expression and the release of PDGF in these platelets and to compare inhibitory effects of abciximab on GPIIb/IIIa mediated fibrinogen binding and inhibition of aggregation with effects on platelet degranulation.

Methods

Study protocol

Nine patients with stable coronary artery disease (CAD) and requiring elective balloon angioplasty were investigated. One patient had one-vessel disease, five patients had two-vessel disease, and two patients had three-vessel disease, respectively. All patients were male and received 100 mg aspirin (Bayer, Germany) daily. The average age was 55.7 years ($\pm$10.9), average weight was 86.3 kg ($\pm$25.4) and the average body mass index was 28.3 ($\pm$7.1). After admittance to the catheter laboratory, the femoral artery was cannulated with a sheath (5F-7F) according to standard fashion using the Judkin approach. A first run in blood sample for assessment of platelet aggregation only was drawn. Patients were then heparinized with a bolus (5000 IU) and subsequent infusion of unfractionated heparin (Roche, Germany) adjusted to obtain a prolongation of the activated clotting time $<$220 s. Angiography was performed with ioversol contrast agent (Mallinckrodt, USA) to identify the stenotic lesions. The average dose of contrast media in these patients was 187$\pm$74 ml. A first blood sample (I) for the full assessment of platelet parameters (see section below) was obtained when the patient was qualified for the PTCA. Abciximab (Lilly, Germany) was administered weight adjusted intravenously (0.25 mg kg^{-1} bolus, followed by a 0125 μg kg^{-1} min^{-1} infusion over 12 h). Balloon angioplasty was performed, all patients received at least one stent and stent deployment was followed by high pressure ($\geq$12 atm) balloon dilatation. Heparin was discontinued immediately after PTCA. 3 h after the start of the abciximab infusion a second blood sample was drawn (II). Then patients received a loading dose of 4×75 mg clopidogrel (Sanofi, Germany). All PTCA procedures were considered to be successful and sheaths were removed at a minimum of 6 h after the discontinuation of abciximab infusion. The last blood sample was obtained 12 h after termination of the abciximab-infusion (III). Each blood sample amounted to 50 ml, which were collected in 10 ml tubes containing in 3.8% sodium citrate (Sarstedt, Germany). Sample I and II were drawn via the sheath, sample III by venipuncture of a large cubital vein through an 18 gauge needle. The study protocol was approved by the institutional review board of the university hospital. All patients gave informed consent for the blood sampling procedure.

Platelet aggregation

Platelet rich plasma (PRP) was prepared by centrifugation of citrated blood at 400 *g* for 3 min at 24°C. Aggregation was performed using a turbidimetric light-transmittance device (APACT Labour, Germany) induced by Adenosindiphosphate (ADP) (Aventis, Germany) at a final concentration of 20 μM and collagen (Nycomed-Amersham, Germany) at a final concentration of 2 μg ml^{-1}. The aggregation response is given as percentage of maximal light transmission [12].

Flow cytometry

Whole blood (250 μl) was diluted and softly mixed 1 : 1 in Hepes-buffer (20 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM Glucose and 1 g l^{-1} bovine serum albumin, pH 7.4). For activation of platelets TRAP (H-Ser-Phe-Leu-Leu-Arg-Asn-Pro-OH, Bachem, Germany) was added at a final concentration of 5 μM and samples were incubated for 10 min at room temperature and fixed with one volume 1% formaldehyde. A further unactivated sample was kept for estimation of the baseline fluorescence intensity. At the end of the activation period 30 μl of fixed platelets were washed with Hepes-buffer by centrifugation for 5 min at 750 ***g***. Platelet sediment was resuspended in 200 μl Hepes-buffer and incubated with saturating concentrations of 10 μl FITC-anti-Fib conjugated antihuman fibrinogen chicken antibodies (FITC-anti h-fibrinogen ex-chicken, WAK-Chemie, Germany), or 10 μl FITC-anti-CD62 (CD62-FITC, IgG1-mouse, Cymbus-Biotechnology LTD, UK) in darkness at room temperature for 30 min. Subsequently samples were incubated with saturating concentration of 20 μl PE-anti CD42b antibody (CD42b-PE, IgG1-mouse, Coulter-Immunotech, France). After incubation with labelled antibodies, samples were diluted further with

1 ml citrate solution 3.8% in D-PBS and centrifuged at 750 *g* for 5 min. Sediment labelled platelets were resuspended in 300 μl − 400 μl of FACS solution for analysis in a FACScan cytometer (Becton Dickinson, USA). Acquisition and processing of data from 5000 platelets were carried out with CONSORT software. Fluorescence channels were set on logarithmic scales. To establish resolution for acceptance of platelet events only binding of the platelet specific PE-labelled anti-CD42b was used to set a gate. Fib-binding and expression of CD62 was measured as mean particle fluorescence intensity (given in arbitrary Units) due to binding of FITC-labelled antibodies to the surface of stimulated platelets [13]. For fibrinogen binding, the difference of the fluorescence intensity between the activated and unactivated platelets obtained with sample I was set to 100% [14]

Preparation of platelets for determination of PDGF

Platelet-derived products preparation was described previously [15] and was slightly modified for this study. The principle of the preparation was to obtain a platelet enriched sample, which contains enough platelets to determine their PDGF-release after stimulation of platelets by thrombin. Platelet rich plasma (PRP) was prepared by centrifugation of citrated blood at 400 *g* for 3 min at 24°C. PRP was then transferred into new tubes. Subsequently one volume (7–10 ml) of PRP (6–8 x 10^9 platelets) was collected into 25% (v/v) acid-citrate-dextrose buffer (citric acid 71 mmol l^{-1}, trisodium citrate 85 mmol l^{-1}, glucose 111 mmol l^{-1}, pH 4,5) and was centrifuged at 300 *g* for 5 min. PRP was collected and centrifuged for 12 min at 1200 *g*. After resuspension in buffer A (14 ml; NaCl 130 mmol l^{-1}, KH_2PO_4 3.9 mmol l^{-1}, Na_2HPO_4 3.9 mmol l^{-1}, NaH_2PO_4 22 mmol l^{-1}, glucose 5.5 mmol l^{-1}, $CaCl_2$ 1 mmol l^{-1}, pH 6,5) platelets were centrifuged at 1200 *g* for 12 min. After this washing step platelets were resuspended in buffer B (tris (hydroxymethyl)aminomethane-HCl 0.02 mol l^{-1} (pH 7.4), NaCl 0.14 mol l^{-1}, glucose 5 mmol l^{-1}, $CaCl_2$ 1 mmol l^{-1}; platelet buffer).

After obtaining the platelet count, the platelet suspension was incubated at 37°C for 2 min and subsequently stimulated with α-thrombin (1 U ml^{-1}, Sigma Chemicals, USA) for 2 min. The proteolytic activity of α-thrombin was then terminated by the addition of 10 U ml^{-1} thrombin-inactivating hirudin (Aventis, Germany). Thereafter, platelet aggregates were removed by centrifugation at 300 *g* for 5 min, followed by a second centrifugation step of 48000 *g* for 30 min. The supernatant was kept and stored at −70°C before use. The amount of $PDGF_{AB}$ in the supernatant was determined by the use of a commercially available immunoassay (human $PDGF_{AB}$, Quantikine, R&D Systems, Germany). There is a 10% cross-reactivity of the $PDGF_{AB}$ immunoassay of the $PDGF_{AB}$ isoform with the $PDGF_{AA}$ and a 2% cross-reactivity with $PDGF_{BB}$.

Statistical analysis

To assess differences between the three sampling points, intraindividual comparison of the data (ADP-and collagen-induced aggregation, fluorescence intensity for fibrinogen and CD62, PDGF-concentration) was carried out following ANOVA and subsequent *U*-test according to Mann & Whitney.

Results

Fibrinogen binding in TRAP-stimulated platelets, as indicated by the mean fluorescence intensity, was significantly reduced from the pretreatment value (i.e. 100%) to 37% in sample II under abciximab infusion and remained reduced to 55% in sample III obtained 12 h after the end of the infusion (Table 1). ADP-induced platelet aggregation was significantly reduced from 86% (sample I) to 37% (II) under abciximab treatment and remained decreased to 54% 12 h after the end of the infusion (III) (Table 1), while collagen-induced aggregation was already low with 44% (due to aspirin pretreatment) and furthermore completely inhibited to 0% in sample II, only slowly recovering in sample III (10%). The aggregation values observed with sample I were not different from those from the run-in sample (Table 1).

Mean fluorescence intensity for CD62 in TRAP-stimulated platelets was only slightly reduced from 78 units (sample I) to 72 units (sample II) and 64 units (sample III). However, the reduction reached statistical significance ($P<0.05$) in sample III (Table 1, Figure 1a).

PDGF secretion from thrombin-stimulated platelets was slightly reduced from 99 ± 33 ng/10^9 platelets in sample I to 82 ± 31 ng/10^9 platelets under abciximab therapy (sample II, NS), and reconstituted to 96 ± 30 ng/10^9 platelets at sample III (Figure 1b). We could not observe any correlation between CD 62 expression in stimulated platelets and secretion of PDGF (Figure 1c).

Discussion

The principal findings of our study were a dissociation between inhibition of GPIIb/IIIa-receptors, the platelet degranulation marker CD62 and granular secretion of PDGF. Although the inhibition of fibrinogen binding and aggregation under abciximab was similar to previously reported values from clinical trials [16, 17] – proving

Table 1 Results of platelet function assessments in nine patients undergoing PTCA under abciximab infusion (mean ± s.d.).

		Sample number		
	Pre-angiography	*I*	*II*	*III*
Aggregation 20 μM ADP (%)	82 ± 12	86 ± 6	37 ± 20*	54 ± 24*
Aggregation 2 μg ml^{-1} collagen (%)	44 ± 31	47 ± 27	0 ± 0*	10 ± 26*
Fibrinogen binding (%)		100	37 ± 34*	55 ± 40*
CD62 (mean fluorescence intensity units)		78 ± 20	72 ± 14	64 ± 12*
PDGF (ng/10^9 platelets.)		99 ± 33	82 ± 31	96 ± 30

§ only aggregation was performed in the run-in sample before angiography. *significantly different from sample I ($P<0.05$).

therefore a therapeutic dosing regimen of abciximab in our patients – CD62 expression was only slightly reduced to a late point in time (sample III). There was only a trend towards a reduced secretion of PDGF under abciximab, however, the PDGF concentrations observed in this study were in agreement with previous reports on CAD patients [18, 19]. We could not demonstrate any correlation of CD62 expression and quantitative PDGF secretion. This is the first report on PDGF-release from platelets obtained *in vivo* during abciximab-therapy.

The growth factor PDGF has sustained effects on vascular remodeling, i.e. proliferation and migration of vascular smooth muscle cells (VSMC) [15, 20] or intima thickening after balloon injury in an animal model [21]. PDGF up-regulates thrombin receptor expression on VSMC [15] and is a strong chemotactic stimulus to fibroblasts and monocytes [22]. It plays an important role in the inflammatory processes of the vascular wall [5]. PDGF is secreted by α-granules of activated platelets. The extent of the degranulation process, which leads to the PDGF release, might be reflected by the expression of the granular membrane protein CD62 (P-selectin) acting as ligand to carbohydrates, e.g. during platelet–monocyte interactions [23]. CD62 expression is enhanced in patients with acute coronary syndromes [6] and elevated CD62 expression is suggested as a predictor of early restenosis after PTCA [7, 24, 25]. Effects of GPIIb/IIIa receptor antagonists on platelet granular secretion of platelet derived products have rarely been investigated [8–11]. However, differences in the antiaggregatory and anti-secretory activities of GPIIb/IIIa inhibitors have been reported, whereby *in vitro* exposure of blood with GPIIb/IIIa inhibitors-resulting in a strong reduction of platelet aggregation and fibrinogen binding by 80–90%-seem to influence secretory activity (CD62 expression, ATP-release) only to a small extent [9, 10]. Gawaz *et al.* [8] reported a slight increase in CD62 expression in patients treated with abciximab, ASA and heparin. In contrast to these observations with GPIIb/IIIa-inhibitors, Rupprecht [26] demonstrated a large and sustained reduction in CD62 expression by 45% in patients after stent implantation for the combination of aspirin and the thienopyridine ticlopidine after 4 weeks treatment. In our study, CD62 expression decreased only at a late point in time (sample III), which might reflect the incoming effect of the loading dose of clopidogrel, which has been initiated between sample II and III. The slight decrease of PDGF under abciximab infusion and increasing levels 12 h after termination suggests a slight and only short lasting inhibitory influence of this GPIIb/IIIa-inhibitor on growth factor release of platelets.

A recent study [27] showed elevated PDFG levels in patients undergoing angioplasty (without GPIIb/IIIa-inhibitors), but a direct correlation between activated platelets and PDGF levels has not been investigated so far. In our study we could not demonstrate an (anticipated) correlation between increase in CD62 expression as marker of α-granular secretion and the secretion of one of the platelet derived products, PDGF. One might speculate that the decrease in CD62 expression at sample III in contrast to restored PDGF levels could indicate that CD62 on the platelet surface is therefore not the ideal marker for platelet degranulation [28].

It must be noted that the stimulus used for PDGF-release in our study was purified human thrombin, in agreement to previous reports [15]. On the other hand, thrombin-receptor activating peptide (TRAP) was required for technical reasons as trigger for flow cytometric detection of CD62 and fibrinogen binding. Thrombin and TRAP shared a similar binding site at the G protein coupled protease-activated receptors (PAR) 1 [29] which accounts for important thrombin signalling in platelets [30]. However, other thrombin binding sites at platelets like PAR 4 and the GP-Ib-IX-V complex are not matched by TRAP [30, 31]. An ideal measurement would have been performed with one sample activated by the same stimulus, but was not feasible in our methodological setting.

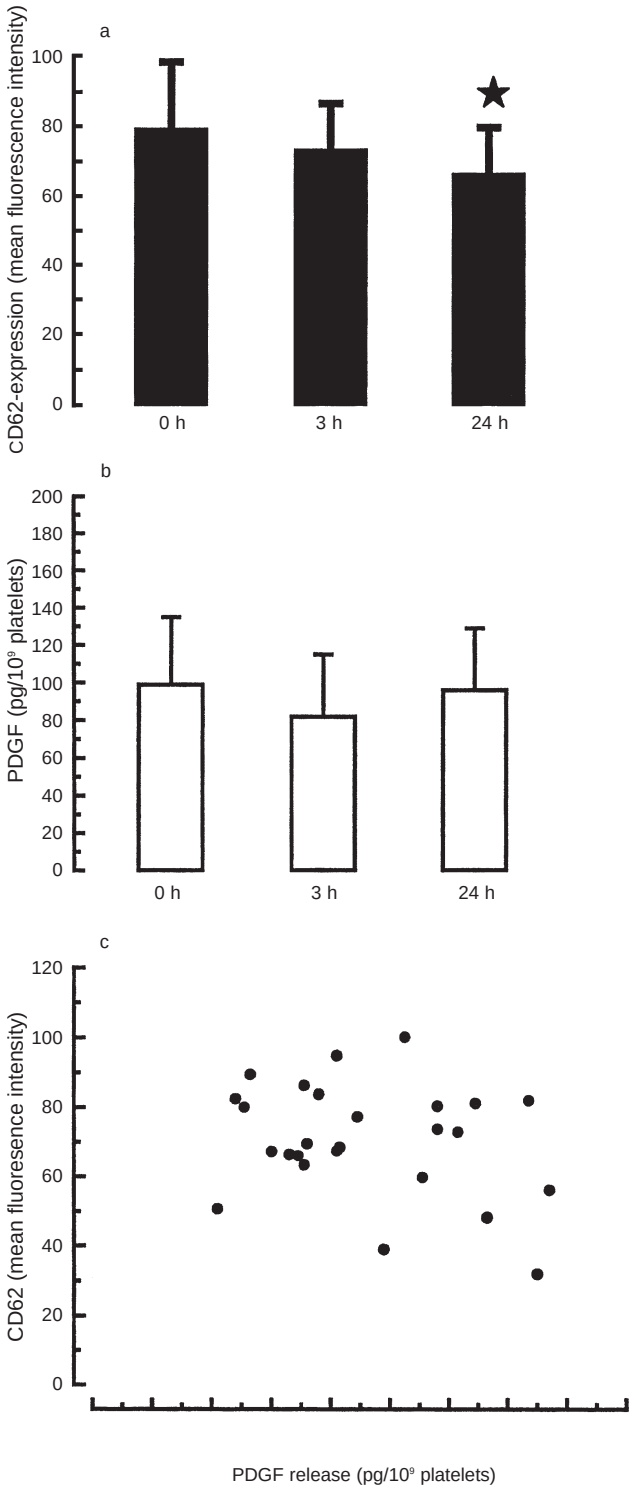

Figure 1 a) TRAP (5 μM)-induced CD62-expression (mean fluorescence intensity), b) PDGF release from platelets induced by 1 U ml^{-1} thrombin. Sample I (0 h, before abciximab), II (3 h, after abciximab) and III (24 h, after abciximab); Mean ± s.d., $n=9$ and c) plot CD62-expression *vs* PDGF-release.

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