Close Relationship between the Platelet Activation Marker CD62 and the Granular Release of Platelet-Derived Growth Factor

JOCHEN GRAFF,1 UTE KLINKHARDT,1 VALÉRIE B. SCHINI-KERTH, SEBASTIAN HARDER, NICOLE FRANZ, STEFFEN BASSUS, and CARL M. KIRCHMAIER

Institute of Clinical Pharmacology, University Hospital, Frankfurt/Main, Germany

Received August 29, 2001; accepted November 6, 2001

This article is available online at http://jpet.aspetjournals.org

ABSTRACT

The expression of CD62 on the surface of platelets is considered to be an indicator of platelet degranulation and secretion. We characterized the relationship between CD62 expression and platelet-derived growth factor (PDGF)AB and PDGFBB secretion in response to thrombin-receptor activating peptide (TRAP). The principal findings were 1) expression of CD62 as a constituent of platelet α-granule membrane and secretion of PDGF, an important ingredient of α-granules, can be stimulated by TRAP-induced activation in a dose-dependent fashion; 2) the activation marker and secretion product are closely correlated with each other; and 3) changes in the CD62 expression induced by a drug, namely clopidogrel, or by a disease, namely diabetes, are paralleled by changes in PDGF secretion. Although CD62 is perceived as an activation marker of platelets indicating enhanced aggregability and secretion of α-granular content, the proof that the CD62 status and its modifications reflect directly the actual secretion of the most important platelet mitogen, PDGF, has so far not been given. This ex vivo-in vitro study shows that at least for the activation pathway provided by the PAR-1 receptor for which TRAP is the selective agonist, CD62 expression on platelets could be a surrogate for their secretory activity.

Platelet secretion of vasoactive factors plays an important role in the development of atherosclerosis (Ross, 1999) and restenosis after coronary interventions (Chandrasekar and Tanguay, 2000). A major mitogenic compound released by aggregating platelets is platelet-derived growth factor (PDGF), which markedly stimulates smooth muscle cell proliferation and migration (Heldin and Westermark, 1999). Neutralizing antibodies to PDGF, and competitive PDGF receptor blocking agents have been shown to inhibit neointimal formation in animal and human studies (Serruys et al., 1997; Bilder et al., 1999; Waltenberger et al., 1999). PDGF exists in three different and biologically active isoforms (AA, BB, AB). Platelets contain mainly PDGFAB and small amounts of PDGFBB, which are stored in α-granules (Hart et al., 1990; Heldin and Westermark, 1999). Activation of platelets, e.g., by thrombin or ADP, is associated with the translocation of CD62 (P-selectin) from the α-granule membrane to the outer surface (Leytin et al., 2000a, b). Once exposed at activated platelets, CD62 allows the interaction of leukocytes with platelets by interacting with leukocyte PSGL-1, thereby triggering inflammatory responses (Furie and Furie, 1995; Evangelista et al., 1996; Zahler et al., 1999).

Flow cytometric determination of CD62 is commonly used to quantify platelet activation status (Hagberg and Lyberg, 2000; Leytin et al., 2000b; Zeiger et al., 2000). The expression of CD62 on the surface of platelets is considered to be an indicator for platelet degranulation and secretion (Gawaz et al., 1996; Michelson et al., 1996; Neumann et al., 1997) and a predictor of acute coronary events (Hollander et al., 1999). It has been shown that upon in vitro activation of platelets, CD62 is detected at the platelet surface, and both α-granule-derived products, like β-thromboglobulin, and dense granule-derived products, like serotonin, are released (Rand et al., 1996). However, the correlation between the surface expression of CD62 and granule content released is still unclear. Moreover, it remains to be determined whether changes in CD62 expression induced by either drugs or diseases are paralleled by changes in the secretion of granule-derived products. Therefore, the aim of the present study was to characterize the relationship between platelet activation status (translocation of CD62) and PDGF secretion from α-granules in response to different concentrations of thrombin-receptor activating peptide (TRAP), and to determine whether such a relationship is maintained under antiplatelet drugs

Supported by the Paul und Cilli-Weil Stiftung, Frankfurt/Main, Germany

1 Contributed equally to this work.

ABBREVIATIONS: PDGF, platelet-derived growth factor; TRAP, thrombin-receptor activating peptide; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; PE, phycoerythrin; PLT, platelet; PRP, platelet rich plasma.
for which it is known that they either reduce CD62 expression (namely, the thienopyridine clopidogrel) (Rupprecht et al., 1998; Klinkhardt et al., 2000) or do not influence CD62 expression (namely the GPIIb/IIIa-inhibitor abciximab) (Fredrickson et al., 2000; Graff et al., 2001), or in clinical conditions that are associated with platelet hyper-reactivity (e.g., diabetes).

Materials and Methods

Chemicals

TRAP (H-Ser-Phe-Leu-Leu-Arg-Asn-Pro-OH) was obtained from Bachem (Heidelberg, Germany). FITC-anti-CDD2 antibody, PE-anti CD42b antibody (IgG1-mouse) and FACS solution for analysis in a FACScan cytometer was obtained from Becton Dickinson (Heidelberg, Germany). PDGFAB was determined by an immunoassay (human PDGFAB; Quantikine, R & D Systems, Wiesbaden, Germany), which has a 10% cross-reactivity with PDGFB and a 2% cross-reactivity with PDGFAB and a limit of sensitivity of 8.4 pg/ml. In several samples, we also determined PDGFBB with a newly available reactivity with PDGF BB and a limit of sensitivity of 8 pg/ml.

Experimental Protocols

The following experimental protocols were performed during this study.

Protocol 1. Relationship between platelet secretion of PDGFAB and CD62 expression in healthy subjects. Blood was drawn from the antecubital vein of male volunteers (n = 6, age 25–41 years) into 3.18% sodium citrate.

Protocol 2. Effect of ex vivo treatment in whole blood with the GPIIb/IIIa-antagonist abicipimab on platelet secretion of PDGFAB and expression of CD62. Blood was drawn from healthy subjects (n = 6, age 22–38 years) as indicated above and spiked with abicipimab (5 µg/ml final concentration). This concentration of abicipimab has been proven to confer >80% inhibition of platelet aggregation and GPIIb/ IIIa-receptor activation (Klinkhardt et al., 2000).

Protocol 3. Effect of in vivo treatment with clopogrel on platelet secretion of PDGFAB and expression of CD62. Blood was drawn from healthy subjects (n = 8, age 25–41 years). Platelet secretion of PDGFAB and expression of CD62 was determined before and after oral administration of clopogrel (loading dose of 2 × 75 mg/day, followed by 75 mg/day for 6 days). Blood was drawn as indicated above.

Protocol 4. Relationship between platelet secretion of PDGFAB and CD62 expression in diabetic patients and age-related healthy control subjects. Patients with noninsulin-dependent diabetes mellitus (n = 8, age 56–71 years) were selected from the diabetes day clinic of the Deutsche Klinik of Diagnostik (Wiesbaden, Germany) and compared with a group of healthy control patients (n = 8, age 59–69 years). Blood was drawn as indicated above.

All clinical protocols have been approved by the Institutional Review Board of the University Clinic of Frankfurt/Main, and written informed consent was obtained. No antiplatelet agent such as aspirin or clopidogrel was taken by the study subjects during the last ten informed consent was obtained. No antiplatelet agent such as aspirin or clopidogrel was taken by the study subjects during the last 4 weeks before the study.

Flow Cytometry Analysis

Citrated whole blood (250 µl) was diluted 1:1 in Hapes buffer (20 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 5.6 mM glucose and 1 g l–1 bovine serum albumin, pH 7.4) and carefully mixed. TRAP at a final concentration of 0 (i.e., unactivated), 1, 2, 5, 10, 20, or 30 µM in the case of protocol 1, and 5 µM for all other protocols, was added to blood samples to activate platelets. Therefore, the sample (30 µl) was washed with Hepes buffer by centrifugation for 5 min at 750g. Platelet sediment was resuspended in Hepes buffer (200 µl) and incubated with saturating concentrations of FITC-anti-CD62 (10 µl) at room temperature for 30 min in darkness. Subsequently samples were incubated with saturating concentration of PE-anti-CDD42b antibody (20 µl), which is used to set a gate for platelet events during the analysis. After incubation with labeled antibodies, samples were diluted with 1 ml of sodium citrate solution (3.8%) in Dulbeco’s phosphate-buffered saline, and centrifuged at 750g for 5 min. The labeled platelet pellets were resuspended in 300 to 400 µl of the FACS solution. Acquisition and processing of data from 5,000 platelets were carried out with CONSORT software (Becton Dickinson).

Statistical Evaluation. Results are presented as mean values ± S.D. Concentration-response curves for the increase in CD62 expression were fitted according to the Akaike criterion. The relationship was assessed by linear regression analysis and Spearman’s rank order correlation coefficient. Differences between observations in samples spiked or not spiked with abicipimab (protocol 2) and in diabetic patients and their controls (protocol 4) were assessed by the Mann-Whitney U test.
Results

PDGF Response to Activation with TRAP (Protocol 1). Platelets activated by TRAP release PDGF in a concentration-dependent manner, and the concentration-response curve for TRAP yielded an EC\textsubscript{50} of 7.9 ± 2.0 μM. Lower concentrations of TRAP (1 and 2 μM) only slightly provoked PDGF\textsubscript{AB} release, compared with unactivated platelets. Maximal PDGF\textsubscript{AB} release was seen at 20 μM TRAP (Fig. 1A). The maximal PDGF\textsubscript{AB} content as determined from platelet lysate (131 ng/10\textsuperscript{9} PLT) was only less above the maximum of the estimated TRAP concentration-response curve. In platelet poor plasma, the amount of PDGF was 4 ± 1 ng/ml. In a subset of samples of protocol 1 and 3, PDGF\textsubscript{BB} release was also investigated. The ratio between the BB and the AB isoform, calculated from all samples was 0.09 ± 0.04. Linear regression analysis showed that the ratio was constant over all concentration levels of PDGF, and therefore seems not to depend on the strength of TRAP as inducer of PDGF release (Fig. 2). Furthermore, the ratio in those samples obtained in protocol 3 under clopidogrel were not different from the samples obtained during protocol 1.

CD62 Expression to Activation with TRAP and Correlation to PDGF Release (Protocol 1). CD62 expression was determined in all samples that were stimulated with TRAP. Differences in the shape of the concentration-response curve were dependent from the analysis of the flow cytometric data. After stimulation with 5 μM TRAP, the expression of CD62 quantified as CD62 positive (% platelets) showed 76% platelets, suggesting a larger effect as when the response is given as MFI, where the increase in MFI from baseline approximated 50% of the maximal response. The EC\textsubscript{50} of TRAP was 2.4 ± 1.0 μM for the CD62 response in % platelets and 4.3 ± 0.7 for MFI, in closer agreement to the EC\textsubscript{50} for release of PDGF (Fig. 1B). Furthermore, since the concentration-response curve for MFI showed a better correlation to the PDGF\textsubscript{AB} release than expression of CD62%+ platelets after various TRAP concentrations ($r^2 = 0.82$ versus 0.74) (Fig. 3), the MFI of CD62 expression has been regarded for reporting the subsequent experiments. From protocol 1, we chose a TRAP concentration of 5 μM as stimulus for platelet activation in the subsequent experiments. At this concentration effects in CD62 expression or PDGF release induced by disease or drugs might be detected in either direction.

Effect of Platelets Spiked in Vitro with Abciximab (Protocol 2). CD62 expression (MFI) was not affected by spiking the blood with abciximab (5 μM/ml) both in unactivated as well as in TRAP-activated samples. PDGF\textsubscript{AB} secretion even showed a slight but not significant increase under abciximab after TRAP activation (29 to 42 ng/10\textsuperscript{9} PLT) (Table 1).

Effect of Clopidogrel on Activation of Platelets (Protocol 3). Baseline values of CD62 expression and PDGF\textsubscript{AB} release were similar at days 1 and 6. After activation with 5 μM of TRAP, a significant decrease ($p < 0.02$) in CD62

Fig. 1. PDGF\textsubscript{AB} release (ng/10\textsuperscript{9} platelets) in PRP and CD62 expression (MFI) in whole blood after activation with TRAP (protocol 1). Mean values ± S.D. ($n = 6$).

Fig. 2. Linear regression analysis (dashed line, 95% confidence interval) of PDGF\textsubscript{AB} release and corresponding PDGF\textsubscript{BB} release in a subgroup of subjects from protocol 1.
expression (238 to 158 MFI), as well as PDGF$_{AB}$ (23 to 12 ng/10^9 PLT), from day 1 to day 6 could be demonstrated (Fig. 4). When the CD62-PDGF data pairs were superimposed to the curve obtained during protocol 1 from a different group of subjects, almost all data were within the 80% prediction interval (Fig. 5).

**Platelet Activation in PRP from Patients with Diabetes Mellitus (Protocol 4).** Compared with age-matched controls, platelets from diabetic patients showed a significantly elevated release of PDGF$_{AB}$ in unactivated PRP (16.4 ng/10^9 PLT versus 9.4 ng/10^9 PLT, p < 0.01) as well as after stimulation with 5 μM of TRAP (45.8 versus 28.7 ng/10^9 PLT, p < 0.03) (Table 1). CD62 expression in unactivated samples was similar in both groups but was significantly enhanced after TRAP stimulation in the diabetes group (276 to 217 MIF, p < 0.03). When the CD62-PDGF data pairs were superimposed to the data obtained from protocol 1, only a few data pairs fell of the 80% prediction interval of the reference curve (Fig. 5).

**Discussion**

The principal findings presented in this paper are: 1) expression of CD62 as a constituent of platelet α-granular membrane and secretion of PDGF, an important ingredient of α-granules, can be stimulated by TRAP-induced activation in a dose-dependent fashion; 2) the activation marker and the secretion product are closely correlated with each other; and 3) changes in the CD62 expression induced by a drug, namely clopidogrel, or a disease, namely diabetes, are paralleled by changes in PDGF secretion. Although CD62 is perceived as an activation marker of platelets indicating enhanced aggregability and secretion of α-granular content, the proof that the CD62 status and its modifications reflect directly the actual secretion of the most important platelet mitogen, PDGF, has not been given so far. This ex vivo-in vitro study shows that at least for the activation pathway provided by the PAR-1 thrombin receptor, for which TRAP is the selective agonist (Kahn et al., 1999), CD62 expression on platelets is a surrogate for their secretory activity. From the stimulus-response curves, we established a concentration of 5 μM TRAP that allows detection of drug- or disease-induced modifications in both directions. On the other hand, patients or treatment groups in our study were not distinguishable on the basis of their baseline secretion of PDGF or their baseline expression of CD62. Samples collected independently from the TRAP dose-response curve obtained from protocol 1 were mostly within the prediction interval (Fig. 5) and suggested a robust and reproducible relationship.

Flow cytometric detection of CD62 expressed on the platelet surface after α-granule release is a common method used to characterize platelet activation in various experimental and clinical conditions (Evangelista et al., 1996; Leytin et al., 2000b). Parameters widely used to describe the activation status are either percentage of CD62 positive platelets in the total platelet population (%+ or the MFI of CD62 positive cells expressed in arbitrary units. Assaying %+ platelets will quantify the proportion of activated cells but will disregard the quantity of expressed platelet surface antibody CD62 (Michelson et al., 2000). MFI represents the mean epitope density of CD62 molecules on the average platelet surface and therefore reflects the activity of the single platelet but not their quantity (Evangelista et al., 1996). In our study, we found a better correlation to PDGF secretion when using the MFI to describe CD62 expression instead %+ platelets. The curve describing the relationship between CD62%+ platelets and PDGF release (Fig. 3, inset) starts extremely shallow, which implies that even upon mild activation a large proportion of the platelet population is detected as %+, but the overall secretion levels remains relatively low. In contrast, at levels of CD62%+ over 80%, PDGF secretion increases 2-fold despite only minor variations in CD62 expression.

PDGF is one of the major mediators for vascular smooth muscle cell proliferation and migration that occur in initial hyperplasia in the process of restenosis and atherosclerosis (Ross 1999). Platelet granules are reported to contain mainly PDGF$_{AB}$ and PDGF$_{BB}$ (Hammacher et al., 1988; Hart et al., 1990). Animal experiments confirmed the role of the B-chain in intimal hyperplasia, especially in preventing vascular smooth muscle cells from apoptosis (Leppanen et al., 2000). Accordingly, the growth activating potency of PDGF$_{BB}$ is reported to be about 4-fold larger than that reported for PDGF$_{AB}$ (Hart et al., 1990). From our data, based on enzyme-linked immunosorbent assay technique, the ratio of the PDGF isoforms AB and BB was approximately 1:10 and not 1:3 as quoted from a recent report employing reversed phase HPLC technique for differential PDGF assay (Hart et al., 1990).

We tried to prove whether the relationship between CD62 expression and secretion of PDGF is maintained under modifications induced by pharmacological or clinical conditions. The thienopyridine P2Y$_{12}$ receptor antagonists are the only antiplatelet drugs that are known to reduce CD62 expression (Rupperecht et al., 1998; Klinkhardt et al., 2000), whereas treatment with GPIIb/IIIa inhibitors (Fredrickson et al., 2000; Klinkhardt et al., 2000), or aspirin (Rinder et al., 1993; Fredrickson et al., 2000; Klinkhardt et al., 2000) did not. We could demonstrate a significant decrease in the platelet activation marker CD62 and the secretion product PDGF after 6
days of clopidogrel treatment. Since it has been observed that via stimulation of phosphatidylinositol-3 kinase the P2Y₁₂ receptor is a necessary constituent for sustained aggregation induced by TRAP (Trumel et al., 1999; Storey et al., 2000), a cross talk between signaling pathways of the PAR-1 thrombin receptor and purinergic receptors is likely and might explain why clopidogrel effects TRAP-induced degranulation.

In diabetic patients, platelet hyper-reagibility is implicated as a risk factor for both microvascular and macrovascular disease (Bern 1978; Barnett 1991) and is associated with an enhanced platelet aggregation response at sites of vascular injury (Winocour 1992) and enhanced CD62 expression on the platelet surface (Tschoepe et al., 1991; Rauch et al., 1999). However, it has not been determined whether platelets from diabetic subjects release more of the content of their granules in response to activation. Although our group of diabetic patients (noninsulin-dependent diabetes mellitus) was small, we observed a significantly higher PDGF-release from platelets after stimulation with TRAP than in age-matched controls. However, CD62 expression and PDGF release did not match in unactivated platelets, which might indicate that a separate mechanism must be taken into account to explain the dissociation between the parameters, at least in patients with noninsulin-dependent diabetes mellitus.

Thrombus-bound platelets at sites of vascular injury could act as a depot for α-granule release of factors like PDGF with sustained effects on the remodeling process (Schini-Kerth et al., 1997). Therefore investigation of platelets activated by a defined stimulus, mimicking the local thrombin response might be closer to the situation of platelet depots in a thrombus than the determination of systemic serum growth factor levels. However, some limitations of our study results need attention. For platelet activation, we used TRAP (SFLL-RNP), which is a synthetic peptide and stimulates the PAR-1 receptor without proteolytic cleavage like the physiological activator thrombin, which also activates PAR-4 and GPIb-receptors (Coughlin 1999; Kahn et al., 1999; Ofosu and Nyarko, 2000). On the other hand, it has been demonstrated that PAR-1 is the primary binding site for human thrombin at platelets, and PAR-1 binding contributes to almost all platelet-activating effects mediated by thrombin (Ofosu and Nyarko, 2000). PDGF itself interferes with platelet activation, and it has been shown in heparinized blood that PDGF₂BB in concentrations of 100 ng/ml reduced TRAP-induced platelet aggregation and platelet microparticle forma-

### Table 1

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 1: before clopidogrel</strong></td>
<td><strong>Day 6: under clopidogrel</strong></td>
<td><strong>Control</strong></td>
</tr>
<tr>
<td>CD62 (MFI)</td>
<td>PDGF (ng/10⁹ Platelets)</td>
<td>CD62 (MFI)</td>
</tr>
<tr>
<td>Unactivated</td>
<td>8.0 ± 2.5</td>
<td>6.3 ± 3.8</td>
</tr>
<tr>
<td>5 μM TRAP</td>
<td>22.7 ± 8.8</td>
<td>11.6 ± 3.0³</td>
</tr>
</tbody>
</table>
| 6.3 ± 3.8 | 95 ± 18 | 158 ± 23³

* p < 0.05 for difference between groups (U test).

* p < 0.05 for difference between day 1 and day 6 (paired t test, two-sided probability).
tion by approximately 20% (Selheim et al., 1999). From our study, the steep shape of the curve describing the relationship between PDGF release and CD62 is determined by data pairs obtained after strong TRAP stimulation. In this part of the curve, the increase in the MFI seems to be terminated already despite a further increase in PDGF, which indeed might indicate a negative feedback of PDGF on platelet activation. Nevertheless, in the above-mentioned study (Selheim et al., 1999), PDGF although reducing platelet aggregation did not affect the expression of CD62.

In conclusion, our data support the utility of flow cytometric determination of CD62 as a target parameter during clinical studies with antiplatelet agents and in acute or chronic vascular diseases. The use of a defined stimulus, as used in common platelet aggregation tests, might allow for detection of drug- or disease-induced modifications and is also closely related to the secretion of the potent mitogen PDGF. However, marked discrepancies in the level of CD62 expression based on the flow cytometric protocol or device used have been described (Serebrany et al., 1999), and the need for standardization of the methodology must be emphasized.

References


Address correspondence to: Prof. Dr. Sebastian Harder, Institute of Clinical Pharmacology, at the Pharmazentrum Frankfurt, University Hospital, Theodor-Stern-Kai-7, D-60590 Frankfurt/Main, Germany. E-mail: harder@em.uni frankfurt.de