Plasma P-Selectin Is Increased in Thrombotic Consumptive Platelet Disorders

By Beng H. Chong, Barbara Murray, Michael C. Berndt, Lindsay C. Dunlop, Timothy Brighton, and Colin N. Chesterman

P-selectin is a 140-kD protein found in the α-granules of platelets and the Weibel-Palade bodies of endothelial cells that on cell activation is expressed on the cell surface and also secreted into the plasma. The secreted form of P-selectin, like plasma P-selectin, differed from platelet membrane P-selectin in that its molecular mass was ~3 kD lower under reducing conditions. Both the secreted and plasma forms of P-selectin contained cytoplasmic sequence as determined by Western blot analysis with an affinity-purified rabbit anti-P-selectin cytoplasmic peptide antibody. We have measured plasma P-selectin and β-thromboglobulin (βTG) concurrently in (1) patients with consumptive thrombotic disorders, including disseminated intravascular coagulation (DIC), heparin-induced thrombocytopenia (HIT), and thrombotic thrombocytopenic purpura (TTP)/haemolytic uremic syndrome (HUS); (2) patients with idiopathic thrombocytopenic purpura (ITP); and (3) healthy controls. Patients with DIC, HIT, and TTP/HUS, but not ITP, had significantly elevated plasma P-selectin and βTG levels when compared with their age-matched healthy controls. The increased plasma P-selectin and βTG in patients with thrombotic disorders were likely to be the result of in vivo platelet and endothelial cell damage or activation. We also found that avoidance of veno-occlusion and other tedious measures customarily taken during blood collection and sample preparation to prevent in vitro platelet activation did not affect plasma P-selectin assay results. In addition, plasma P-selectin levels were not influenced by the presence of renal failure or heparin administration. These results indicate that plasma P-selectin may be a useful new marker for thrombotic diseases.

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DIC. Twenty-one patients with DIC were studied, including 4 males and 7 females, with ages ranging from 24 to 99 years (mean, 58 years). They had reduced plasma fibrinogen (mean ± SD, 1.2 ± 0.6 g/L), elevated d-dimer (mean ± SD, 1.8 ± 0.6 mg/L), thrombocytopenia (mean platelet count ± SD, 63 ± 30 × 10^9/L), prolonged activated partial thromboplastin time (aPTT) and/or prothrombin times, and a predisposing cause for DIC, which included sepsis, cancer, heartstroke, and obstetric accidents.14

HIT. Twenty-one patients with HIT (8 males and 13 females) whose ages ranged from 39 to 83 years (mean age, 58 years) were studied. They fulfilled the following diagnostic criteria: (1) a normal platelet count before heparin treatment; (2) thrombocytopenia (mean platelet count ± SD, 58 ± 28 × 10^9/L) occurring during heparin therapy; (3) exclusion of other causes of thrombocytopenia; and (4) a heparin-dependent antiplatelet antibody detected in the patients’ sera/plasma by platelet aggregometry.15 Twelve patients had acute arterial and/or venous thrombosis.

TTP/HUS. Three patients with TTP and 2 with HUS (1 male and 4 females) were included in the study. Their ages ranged from 5 to 56 years (mean, 48 years). They had thrombocytopenia, microangiopathic hemolytic anemia, and impaired renal function. In addition, the patients with TTP had fever and neurologic manifestations such as coma and fits.14 The patients with TTP had been treated with aspirin and dipyridamole before the blood was sampled.

Patients With Idiopathic Thrombocytopenic Purpura (ITP)
Twenty-one patients with ITP (10 males and 11 females) were studied as controls because in vivo platelet activation and endothelial cell perturbation is uncommon in this disorder. Their ages ranged from 21 to 94 years (mean, 52 years). They had a nonpla-
sable spleen, normal or increased number of megakaryocytes in the bone marrow aspirate, and a thrombocytopenia for which all other causes were excluded by clinical features and laboratory investigations.16

Patients With Chronic Renal Failure
Twenty-four patients with stable chronic renal failure between the age of 25 to 92 years (mean, 54 years) were studied. They had varying degrees of renal impairment, with plasma creatinine ranging from 0.12 to 1.25 mmol/L, normal platelet counts, and no clinical thrombosis.

Healthy Controls
Thirty-nine healthy controls (22 females and 17 males) were studied. Their ages ranged from 22 to 90 years (mean, 55 years), with 23 below and 14 above 60 years of age.

Blood Collection and Plasma Preparation
Blood was collected from patients and controls without veno-occlusion, using a 19-gauge butterfly needle and two-syringe technique, as previously described.15 The blood was collected in polypropylene tubes containing 0.5 mL of anticoagulant mixture, ETP (110 mmol/L disodium EDTA, 12 mmol/L theophylline, and 2.82 μmol/L prostaglandin E1 [PGE1]). The blood was immediately kept cold in melting ice until it was centrifuged and the plasma was removed. To investigate the influence of blood collection and preparation procedures on plasma P-selectin assay levels, in some experiments a certain step(s) in the above procedure was deliberately omitted or altered. Additionally, blood was processed in the same manner as platelet-poor plasma would be prepared for routine blood coagulation tests, as previously described.15

Informed consent for blood collection was obtained from every patient and normal volunteer. All subjects, except the patients with TTP, did not take any medications that may inhibit platelet function for at least 10 days before their participation in the study.

Polyclonal Antibodies
The preparation of an affinity-purified rabbit polyclonal antihuman P-selectin antibody has been previously described in detail.14,15 Antipeptide antibodies were raised in New Zealand white rabbits using peptide-keyhole limpet hemocyanin (KLH) conjugates. Peptide sequences selected from the transmembrane (exon 14) and cytoplasmic domains (exon 15) of P-selectin were made to specification by Auspep Pty Ltd (Melbourne, Australia). The transmembrane domain peptide was CALLRRKROK, with the N-terminal cysteine residue added to allow coupling to KLH. The cytoplasmic domain peptide was CPLLPSHSLGT. Both peptides contained free N- and C-termini. Peptides were coupled to KLH using m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), as previously described.16 For the initial subcutaneous injection, the peptide-KLH conjugate (1 mg in 1 mL) was mixed with Freund’s complete adjuvant. At 3-weekly intervals, two booster innoculations were similarly performed, but with Freund’s incomplete adjuvant. Antipeptide antibodies were affinity-purified using a 1 × 10 cm column of peptide-bovine serum albumin conjugate coupled to a mixture of Affigel 10 and 15 (Bio-Rad, Richmond, CA). Bound antibody was eluted with 0.1 mol/L glycine, pH 2.4, and immediately neutralized by the addition of 0.2 vol of 1 mol/L Tris, pH 8.0. The eluted Ig was dialysed exhaustively against 0.01 mol/L Tris, 0.15 mol/L sodium chloride, pH 7.4, and stored at -70°C.

P-Selectin and βTG Assays
The P-selectin assay was performed using a sandwich enzyme-linked immunosorbent assay (ELISA) and the plasma βTG assay was performed using a rabbit anti-βTG antibody, both as previously described in detail.15 The latter was performed on the same plasma samples on which P-selectin was assayed.

Plasma samples for βTG and P-selectin assays were collected within 3 days of diagnosis of DIC, HIT, TTP/HUS, and ITP; in 5 of these patients with HIT, serial samples were also collected at regular intervals for about 10 days.

Pulse Radiiodination of α-Thrombin-Treated Platelets
Washed platelets were α-thrombin activated and pulse radioiodinated essentially as previously described.20 Briefly, washed platelets were activated with α-thrombin (1 U/mL, final concentration) in the presence of Na^121I (1 mcg/mL) and lactoperoxidase (2.5 μg/mL), and 30 seconds later the platelet suspension was made 35 μmol/L in hydrogen peroxide. After a further 30 seconds, the radioiodination was terminated by the addition of catalase (3.5 μmol/L, final concentration). The platelets were then pelleted by centrifugation at 1,000g for 15 minutes. The supernatant was recentrifuged at 8,370 g for 5 minutes and then at 100,000g for 3 hours. The platelet pellet was resuspended at 10^9 platelets/mL in HEPES buffer for immunoprecipitation analysis.
Analytical Methods

SDS-polyacrylamide gel electrophoresis (SDS-PAGE), immunoprecipitation analysis, and Western blot analysis were performed as previously described in detail.\textsuperscript{19,20}

Statistical Analysis

Statistical analysis was performed, unless otherwise stated, using the Mann Whitney U test. Spearman’s rank correlation test was used to analyze the relationship between plasma P-selectin and $\beta$TG, and, in patients with chronic renal failure, between plasma P-selectin and creatinine and plasma $\beta$TG and creatinine. The results were expressed as medians and absolute ranges, with the latter in parenthesis.

RESULTS

Plasma/Secreted Form of P-Selectin

We have previously shown that a soluble form of P-selectin circulates in plasma and can be secreted from activated platelets.\textsuperscript{4} On SDS-polyacrylamide gels, the plasma form of P-selectin had an identical molecular mass to platelet membrane P-selectin under nonreducing conditions, but was $\sim 3$ kD smaller under reducing conditions. Analytical gel filtration analysis indicated that the plasma form of P-selectin eluted as a monomer, whereas detergent-free, platelet membrane P-selectin eluted primarily as a tetramer. Both these findings are consistent with plasma P-selectin lacking an hydrophobic transmembrane sequence and are consistent with its potential derivation from the alternatively spliced form of P-selectin lacking exon 14 that encodes the transmembrane domain.\textsuperscript{3,6} Both platelets and endothelial cells have been shown to contain mRNA encoding the soluble form of P-selectin.\textsuperscript{6} In this study, additional experiments were performed to further characterize the platelet-secreted and plasma forms of P-selectin.

Because the rabbit affinity-purified anti–P-selectin antibody does not recognize reduced P-selectin after SDS-PAGE, we pulse radioiodinated platelets between 30 and 60 seconds after the addition of $\alpha$-thrombin as a platelet stimulus. Immunoprecipitation of the washed and detergent-lysed platelet pellet with polyclonal anti–P-selectin antibody demonstrated that P-selectin had been surface expressed within the first minute of platelet activation (Fig 1A, lane 2). Radiolabeled P-selectin could also be immunoprecipitated from the ultracentrifuged supernatant of these thrombin activated platelets (Fig 1A, lane 3). As is the case for plasma P-selectin, the P-selectin in the ultracentrifuged supernatant from these platelets had a lower molecular mass ($\sim 3$ kD) under reducing conditions than the P-selectin that was platelet membrane associated (Fig 1A), although the molecular size was similar under nonreducing conditions (data not shown and Dunlop et al).\textsuperscript{4}

A polyclonal antibody directed against a P-selectin cytoplasmic peptide (sequence within that defined by exon 15) Western-blotted purified platelet membrane P-selectin under nonreducing and reducing conditions (data not shown)
and P-selectin in whole platelets (Fig 1B, lane 1). An additional band of unknown derivation was seen at ~95 kD. This antibody recognized the secreted form of P-selectin in the platelet supernatant both before (Fig 1C, lane 2) and after ultracentrifugation of the supernatant at 100,000g for 3 hours (Fig 1C, lane 3). Interestingly, although only ~15% of the total platelet P-selectin pool is secreted on activation (calculated from Dunlop et al14), the anti-P-selectin cytoplasmic peptide antibody reacted more strongly with secreted P-selectin than with the P-selectin, which remained platelet associated (Fig 1C, lanes 2 and 3 v lane 1). This difference in immunoreactivity versus relative amount was also observed with purified plasma P-selectin, which also reacted more strongly than platelet membrane P-selectin with this antibody (data not shown). Because there are only 11 intervening amino acids in the P-selectin sequence between the cytoplasmic peptide used as the immunogen and the putative transmembrane spanning peptide,1 the difference in immunoreactivity may reflect relative epitope access on nitrocellulose depending on whether the transmembrane domain encoded by exon 14 is present or not. Unfortunately, the antipeptide antibody directed against the peptide sequence within exon 14 did not recognize intact platelet membrane P-selectin and therefore could not be used to fully define the derivation of the secreted and plasma forms of P-selectin.

Plasma Concentrations of P-Selectin in Healthy Controls and Patients

The plasma levels of P-selectin and βTG of 37 healthy controls are shown in Fig 2. The median plasma P-selectin value of the healthy subjects was 0.21 μg/mL (range, 0.09 to 0.40 μg/mL) and the median plasma βTG level was 18.3 ng/mL (range, 9.0 to 39.5 ng/mL). Plasma P-selectin and βTG levels of the healthy controls aged more than 60 years were significantly higher than those aged less than 60 years. However, there was no significant difference in plasma P-selectin or βTG values between the male and female subjects.

As shown in Fig 2, plasma P-selectin and βTG levels of the patients with DIC, HIT, and TTP/HUS were substantially higher than those of the age-matched healthy subjects, with little overlap between the patients’ and the controls’ values. In contrast, the plasma P-selectin and βTG values of
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P-selectin plasma levels were insensitive to collection procedures or speed of centrifugation. P-selectin plasma values did not differ from those of healthy controls, and the omission of platelet dust did not cause an increase in P-selectin values. Plasma P-selectin and creatinine levels were closely correlated (Spearman’s test, r = 0.7, P < 0.005). Serial measurements were performed on 5 patients with HIT and the results of a representative patient are shown in Fig 3. The plasma P-selectin and βTG levels were initially markedly increased, but decreased to normal values in about 11 days after cessation of heparin and commencement of antithrombotic therapy with Orgaran (a new low molecular weight heparinoid) and warfarin.

Experiments were also performed to investigate whether measures customary taken to prevent in vitro platelet activation and sample contamination by platelets/platelet dust are necessary for assays of plasma P-selectin. Both P-selectin and βTG were assayed in plasma from 10 healthy subjects prepared with these preventive measures and compared with the plasma of the same subjects prepared without these measures selectively omitted. It was found that the omission of any one of the measures, i.e., (1) potent platelet inhibitors (ETP), (2) maintenance of the sample temperature at 0°C to 4°C, and (3) ultracentrifugation or centrifugation three times at 12,300g for 5 minutes with sample temperature kept throughout at 0°C to 4°C. With each of the alternative procedures, a certain step was modified as indicated above. P-selectin, βTG, and platelet concentrations were measured in each plasma sample and the results obtained with each alternative procedure were compared with those obtained with the standard procedure (1) (control values). Each result above represents the median with the range in parenthesis.

<table>
<thead>
<tr>
<th>Preparative Procedures</th>
<th>Centrifugation</th>
<th>Anticoagulant</th>
<th>Temperature</th>
<th>Veno-Oclusion</th>
<th>Platelets</th>
<th>P-Selectin</th>
<th>βTG</th>
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Blood was collected from 10 healthy subjects and plasma prepared using a standard procedure (1) as described in Subjects and Methods or an alternative procedure (2 through 7). With the standard procedure, blood was collected without veno-occlusion into ETP (EDTA, theophylline, PGE,) and plasma prepared by centrifugation three times at 12,300g for 5 minutes with sample temperature kept throughout at 0°C to 4°C. With each of the alternative procedures, a certain step was modified as indicated above. P-selectin, βTG, and platelet concentrations were measured in each plasma sample and the results obtained with each alternative procedure were compared with those obtained with the standard procedure (1) (control values). Each result above represents the median with the range in parenthesis.

The patients with ITP did not differ from those of the healthy controls. There was a close correlation between the plasma P-selectin and βTG values (Spearman’s test, r = 0.851, P < 0.005). Serial measurements were performed on 5 patients with HIT and the results of a representative patient are shown in Fig 3. The plasma P-selectin and βTG levels were initially markedly increased, but decreased to normal values in about 11 days after cessation of heparin and commencement of antithrombotic therapy with Orgaran (a new low molecular weight heparinoid) and warfarin.

Experiments were also performed to investigate whether measures customary taken to prevent in vitro platelet activation and sample contamination by platelets/platelet dust are necessary for assays of plasma P-selectin. Both P-selectin and βTG were assayed in plasma from 10 healthy subjects prepared with these preventive measures and compared with the plasma of the same subjects prepared with one of these measures selectively omitted. It was found that the omission of any one of the measures, i.e., (1) potent platelet inhibitors (ETP), (2) maintenance of the sample temperature at 0°C to 4°C, and (3) ultracentrifugation or centrifugation three times at 12,300g for 5 minutes to remove platelets/platelet dust, resulted in a significant increase of βTG values (Table 1). In contrast, omission of these measures did not cause an increase in P-selectin values. Plasma samples processed without these measures and, in the same manner as samples for routine coagulation tests, gave P-selectin values that did not differ significantly from those processed with these measures (P > 0.01). This finding is in agreement with that of Dunlop et al., who also found that P-selectin plasma levels were insensitive to collection procedure or speed of centrifugation.

To investigate whether renal failure affects plasma concentrations of βTG and P-selectin, plasma levels of these proteins were measured in 24 patients with varying degrees of renal failure but without clinical evidence of thrombosis or platelet consumption. There was no correlation between plasma P-selectin and creatinine levels (Spearman’s test, r = 0.206, P > 0.1), whereas plasma βTG and creatinine values were closely correlated (Spearman’s test, r = 0.641, P < 0.002) (Fig 4). In addition, plasma P-selectin levels were found to be unaffected by heparin administration. There was no significant increase in plasma P-selectin when heparin (5,000 IU) was injected intravenously into 3 subjects (Table 2).

DISCUSSION

A previous study from our laboratory showed that P-selectin was secreted from activated platelets and also circulated in plasma at a concentration of ~2 μg/mL. In this study, we found that the secreted form of P-selectin, similar to plasma P-selectin, differed from platelet membrane P-selectin in that it had a slightly lower molecular mass by ~3 kD under reducing conditions. Both the plasma and secreted forms of P-selectin immunoblotted with an anti-P-selectin cytoplasmic peptide antibody, excluding the possibility that they are derived by proteolysis of the extracellular portion of platelet membrane P-selectin. P-selectin is known to be a major constituent of platelet dust or microparticles, which are formed on platelet activation and also circulate in plasma. However, the following evidence suggests that the secreted and plasma forms of P-selectin we have described are probably not associated with platelet membrane microparticles. First, as discussed above, both these forms of P-selectin are smaller in molecular weight than platelet membrane P-selectin. Second, the concentrations of these forms of P-selectin in solution are unaffected.

Table 1. P-Selectin, βTG, and Platelet Concentrations in Plasma Prepared by Different Procedures and With Different Anticoagulants

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* Not significantly different from control values.
† Significantly different from control values (P < .001).
by ultracentrifugation at 100,000g for 3 hours, conditions previously reported to sediment platelet microparticles. Third, the calculated level of P-selectin that would be microparticle-associated in plasma is three orders of magnitude less than the amount detected by ELISA. Finally, if the affinity-purified polyclonal anti-P-selectin antibody used for coating the solid phase in the P-selectin ELISA was used satisfactorily for the plasma P-selectin ELISA, MoAbs against GP IIb-IIIa or GP Ib-IX give only background measurements (Dunlop and Berndt, unpublished observations).

Given that platelets contain mRNA-encoding soluble P-selectin, it is probable that the secreted and plasma forms of P-selectin represent the alternatively spliced, soluble form of P-selectin lacking the transmembrane sequence encoded by exon 14.

However, several lines of evidence argue against this being the only explanation of our findings. Firstly, the slight smaller molecular weight could be accounted for by proteolytic cleavage from either the N- or C-terminus of membrane P-selectin or by slight differences in posttranslational modifications such as decreased glycosylation. Secondly, microparticles do not quantitatively sediment in undiluted plasma even though a previous study did show sedimentation of microparticles after diluting the plasma. Thirdly, some platelet perturbations such as complement fixation can generate microparticles relatively enriched in P-selectin. Finally, the alternatively spliced form of P-selectin transfected into ALT 20 cells is secreted and not packaged into the storage granules. Considering together the available evidence, it is possible that the soluble form of P-selectin in plasma could coexist with P-selectin on microparticles, but the relative proportions of each form need to be further investigated.

In addition to characterization of the secreted and plasma forms of P-selectin, we also studied plasma P-selectin in conjunction with βTG in healthy individuals and patients with consumptive platelet disorders. We observed among the healthy controls slightly higher plasma P-selectin and βTG concentrations in those more than 60 years of age, consistent with a previous report that found that plasma βTG levels increased with age. These observations might possibly be explained by the higher incidence of subclinical vascular disease in apparently healthy elderly individuals. However, the more striking finding, which has not previously been reported, was the significant and often marked increase in plasma P-selectin levels that occurred in parallel with increased plasma βTG levels in patients with DIC, TTP/HUS, and HIT. There was little overlap between the values of these patient groups and the age-matched healthy control group. The increased plasma P-selectin and βTG levels in patients with these thrombotic disorders may be the result of activation or damage of platelets and endothelial cells. In contrast, the lack of increased plasma P-selectin and βTG in patients with ITP may reflect the infrequent occurrence of in vivo platelet activation and endothelial cell perturbation in this condition.

Additionally, we found that plasma P-selectin measurement, unlike plasma βTG and PF4 assays, was not influenced by omission of the cumbersome measures customarily taken to prevent in vitro platelet activation during blood collection and sample preparation. Furthermore,
plasma P-selectin concentrations were not increased by intravenous heparin administration, which is known to increase plasma PF4 concentrations by about 30-fold, nor were they affected by renal insufficiency, which is known to increase plasma P-selectin concentrations. Our overall results and the recent observations by Wu et al. of increased plasma P-selectin in patients with acute myocardial infarction and stroke suggest that plasma P-selectin is a convenient and useful marker for thrombotic diseases, particularly those with platelet/endothelial cell activation or damage.

ACKNOWLEDGMENT

We thank Dr. Richard Fisher for his assistance in statistical analysis of the data and Bernadette O'Reilly for typing the manuscript.

REFERENCES

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