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SHORT COMMUNICATION

Microparticle-associated P-selectin reflects platelet activation in preeclampsia

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Abstract
Platelet activation in preeclampsia is reflected by elevated levels of platelets exposing P-selectin. In plasma, a non-cell bound (soluble) form of P-selectin is present. Elevated levels of this soluble form have been reported in preeclampsia. Plasma P-selectin may consist of two fractions: microparticle (MP)–associated P-selectin and non-MP–associated P-selectin. In the present cross-sectional study, we investigated to which extent plasma P-selectin is MP–associated and whether such MP are elevated in preeclamptic patients. Preeclamptic patients ($n = 10$) were matched with normotensive pregnant women ($n = 10$) and non-pregnant controls ($n = 10$). Plasma P-selectin was measured by ELISA. MP were isolated, double labelled with anti-CD61 (GPIIIa) and anti-CD62P (P-selectin) and subsequently analyzed with flowcytometry. Plasma P-selectin concentration was elevated in preeclamptic patients compared to non-pregnant controls ($p = 0.007$), but not compared to normotensive pregnant women ($p = 0.210$). Plasma P-selectin is partially MP–associated (3–5%). In pregnancy, the fraction of P-selectin exposing platelet-derived MP (PMP) (10.9%) was increased compared to non-pregnant controls (8%). This fraction further increased in preeclamptic patients (15.4%), and significantly differed from normotensive pregnant women ($p = 0.02$). A minor fraction of plasma P-selectin is associated with PMP. The fraction of PMP exposing P-selectin is increased in preeclamptic patients and to a lesser extent in normotensive pregnancy. Because MP associated P-selectin exclusively originates from platelets, this fraction indicates platelet activation. Platelet activation is prominent in preeclampsia and this study proves that at least a part of the plasma P-selectin originates from platelets.

Keywords: *Microparticles, platelet activation, P-selectin, preeclampsia*

Introduction

Platelet activation is evident in preeclamptic patients as indicated by an elevated number of platelets exposing P-selectin [1–3]. Also elevated levels of plasma P-selectin have been reported in preeclamptic patients [4–8]. This plasma P-selectin can theoretically consist of two fractions: Microparticle (MP)-associated P-selectin and non-MP-associated P-selectin. MP are small (less than 1 μm) procoagulant membrane vesicles released from blood cells or endothelial cells on activation or during apoptosis. In human blood, cell-derived MP predominantly originate from platelets. Platelet-derived MP (PMP) are indisputably related to coagulation [9]. Although elevated levels of PMP have been reported in patients with hypertension and acute coronary disease [10, 11], they are not increased in preeclamptic patients [12]. Yet, there is evidence that MP are responsible for the impairment of endothelial function in preeclampsia [13]. MP associated P-selectin could contribute to the damaging action of MP in preeclampsia. It is unknown to what extent plasma P-selectin is associated with PMP in preeclamptic patients and controls. Therefore, we investigated the fraction of MP-associated P-selectin in plasma in preeclamptic patients, healthy normotensive pregnant women and non-pregnant controls and the numbers of P-selectin-exposing PMP in these patients.

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Materials and methods

Patients

Blood samples were obtained from preeclamptic patients ($n = 10$), normotensive pregnant women ($n = 10$) and non-pregnant controls ($n = 10$). The women were matched for age (± five years) and parity. The preeclamptic patients and normotensive pregnant women were also matched for gestational age (± two weeks). Inclusion criteria for preeclampsia were: (1) diastolic blood pressure of 110 mm Hg or more on any occasion or 90 mm Hg or more on two separate occasions at least four hours apart, (2) proteinuria of at least 0.3 g protein/24 hours and (3) symptoms developing after 20 weeks gestational age in a previously and subsequently normotensive woman. Patients with only gestational hypertension or intra-uterine growth retardation or patients using medication other than antihypertensive treatments, were excluded. The control groups consisted of healthy women not using any medication including oral contraceptives.

Collection of blood samples

Two blood samples were taken from the antecubital vein without tourniquet through a 20-gauge needle with a vacutainer system. The first sample was collected into a 4.5 mL tube containing 0.105 M buffered sodium citrate (Becton Dickinson, San Jose, CA). Within 30 minutes after collection, cells were removed by centrifugation for 20 minutes at 1560 *g* and 20°C. Plasma samples were then divided in 250 μL aliquots, immediately snap frozen in liquid nitrogen and stored at −80°C. The second sample was collected in a 4.5 mL tube containing ethylenediaminetetraacetic acid (EDTA; Becton Dickinson; San Jose, CA) for determination of the total number of platelets.

Reagents and assays

Fluorescein isothiocyanate (FITC)-labelled IgG_1 and phycoerythrin (PE)-labelled IgG_1 were obtained from Becton Dickinson (San Jose, CA), anti-CD61-FITC from Pharmingen (San Jose, CA) and anti-CD62P-PE from Immuno Quality Products (Groningen, The Netherlands). Finally, allophycocyanin (APC)-conjugated annexin V was purchased from Caltag (Burlingame, CA). The following final dilutions of antibodies were used: IgG_1-FITC (1:10), IgG_1-PE (1:10), anti-CD61-FITC (1:30), anti-CD62P-PE (1:10) and annexin V-APC (1:40). Plasma concentrations of P-selectin were determined using enzyme-linked immunosorbent assays (ELISA). Assays were performed as described by the manufacturer (Parameter human sP-Selectin Immunoassay by R&D Systems; Minneapolis, USA). Platelet counts were determined with a Cell-Dyn 4000 (Abbott Diagnostics Division; Abbott Laboratories; Hoofddorp, The Netherlands) at the department of Clinical Chemistry (Academic Medical Center; Amsterdam, The Netherlands).

Isolation of microparticles

A sample of 250 μL frozen plasma was thawed on ice and centrifuged for 30 minutes at 17 570 *g* and 20°C to pellet the MP. After centrifugation, 225 μL of the supernatant was removed. The MP pellet and remaining supernatant were resuspended in 225 μL phosphate-buffered saline with citrate (154 mmol/L NaCl, 1.4 mmol/L phosphate, 10.9 mmol/L trisodium citrate, pH 7.4). After centrifugation for 30 minutes at 17 570 *g* and 20°C, 225 μL of the supernatant was removed again. The MP pellet was then resuspended in 75 μL PBS-citrate.

Flowcytometry

Monoclonal antibodies directed against glycoprotein (GP) IIIa (CD61) and P-selectin (CD62P) were used. Five μL of the MP suspension was diluted in 35 μL $CaCl_2$ (2.5 mmol/L)-containing PBS. Then 5 μL APC-labeled annexin V was added to all tubes plus 5 μL of the cell-specific monoclonal antibody or isotype-matched control antibodies. The samples were then incubated in the dark for 15 minutes at room temperature. After incubation, 900 μL of calcium-containing PBS was added to all tubes (except to the annexin V control, to which 900 μL citrate-containing PBS was added). Samples were analysed for one minute in a fluorescence automated cell sorter (FACS Calibur) with CellQuest software (Becton Dickinson, San Jose, CA). Both forward scatter (FSC) and sideward scatter (SSC) were set at logarithmic gain. MP were identified on basis of their size and density and on their capacity to bind annexin V. Annexin V measurements were corrected for autofluorescence. Labelling with cell-specific monoclonal antibodies was corrected for identical concentrations of isotype-matched control antibodies.

Statistical analysis

Data were analysed with Statistical Package of the Social Science software for Windows, release 11.5 (SPSS Benelux BV, Gorinchem, The Netherlands). Demographic data are presented as medians with the minimal and maximal value and analysed with Mann-Whitney U Tests. The data of the flowcytometric analysis and ELISA were analysed with Kruskall Wallis tests for differences among three groups and Mann-Whitney U tests for differences between two groups. A probability value of <0.05 was considered statistically significant.

Correlations were calculated with a two-sided bivariate Pearson correlation test.

Results

Patient characteristics

Patient characteristics are summarized in Table I. As expected, birth weight and gestational age at delivery were significantly lower in the preeclamptic women and both systolic and diastolic blood pressures were significantly higher compared to normotensive pregnant women and the non-pregnant controls. One of the preeclamptic women developed HELLP (hemolyse, elevated liver enzymes, low platelets) syndrome. Analyses were also performed without the HELLP patient. One preeclamptic patient was excluded from the MP analysis because MP were hardly detectable due to loss of the MP pellet during the isolation procedure.

Levels of P-selectin fractions in plasma

The concentration of P-selectin in cell-free plasma of nine non-pregnant controls fell within the normal range (18–40 ng/mL), as did seven normotensive pregnant women and four preeclamptic patients (Figure 1A). The median concentration of plasma P-selectin in the preeclamptic group was 51.0 ng/mL which differed significantly from the non-pregnant controls (22.8 ng/mL, $p = 0.007$), but not from the normotensive pregnant women (35.5 ng/mL, $p = 0.1$). Three to five per cent of the plasma P-selectin could be removed by high-speed centrifugation, indicating that this fraction is MP-associated. This percentage did not differ significantly among groups.

Platelets and MP

Whole blood platelet counts (Table II) were significantly lower in the preeclamptic group compared to normotensive pregnant women and non-pregnant controls. Only one patient developed HELLP syndrome, but after excluding this patient, the number of platelets was still significantly lower in the preeclamptic group. The majority of MP (85–98%) stained for CD61, indicating that they originated from platelets. The numbers of MP and PMP were comparable between the control groups, but reduced in preeclamptic patients. The ratio between PMP counts and platelet numbers was consistent (0.02) in the three groups studied; suggesting a direct association between the number of circulating platelets and the number of PMP. Therefore, the lower number of PMP in the preeclamptic patients most likely reflects the lower number of circulating platelets. The fraction of PMP exposing P-selectin was increased in preeclamptic patients compared to normotensive pregnant women (15.4% vs. 10.9%; $p = 0.02$) and compared to non-pregnant controls (8.0%; $p = 0.001$). In Figure 1B the percentages of PMP exposing P-selectin, for all individuals are presented. No differences were found in the mean fluorescence intensity of the P-selectin-exposing subpopulations of PMP among groups, suggesting that the quantity of P-selectin exposed per MP was comparable (data not shown).

Discussion

A minor fraction of plasma P-selectin is associated with MP. P-selectin is present both in Weibel-Palade bodies of endothelial cells and in the α-granules and dense bodies of platelets. This implies that P-selectin

Table I. Patient characteristics.

	Preeclampsia ($n = 10$)	Normotensive pregnancy ($n = 10$)	Non-pregnant controls ($n = 10$)	p	$p^\star$
Age (years)	30.6 (20.8–35.0)	31.5 (21.4–39.2)	30.0 (20.0–36.0)	NS	NS
Gestational age					
At study (weeks)	29.4 (24.8–32.1)	29.6 (24.9–32.7)	–	NS	–
At delivery (weeks)	33.9 (26.1–36.1)	39.0 (36.1–42.1)	–	0.001	–
Blood pressure					
Systolic (mmHg)	163 (140–220)	110 (100–120)	108 (95–133)	0.0001	0.0001
Diastolic (mmHg)	115 (95–120)	65 (60–75)	69 (65–85)	0.0001	0.0001
BMI (kg/m^2)	25.8 (20.0–51.9)	22.2 (19.7–31.9)	–	NS	–
Proteinuria (g/L)	3.9 (0.37–6.97)	–	–	–	–
SGOT (U/L)	33.5 (19–310)	–	–	–	–
LDH (U/L)	259 (149–912)	–	–	–	–
Parity					
Primiparous	7	7	7	–	–
Multiparous	3	3	3	–	–
Birth weight (g)	1160 (470–2090)	3515 (2490–3870)	–	0.001	–

Data are presented as median (range). BMI: Body Mass Index, SGOT: serum glutamate oxaloacetate transaminase, LDH: lactate dehydrogenase. p: Statistical difference between the preeclamptic patients with the normotensive pregnant women. $p^\star$: Statistical difference between the preeclamptic patients and non-pregnant controls. NS: not significant.

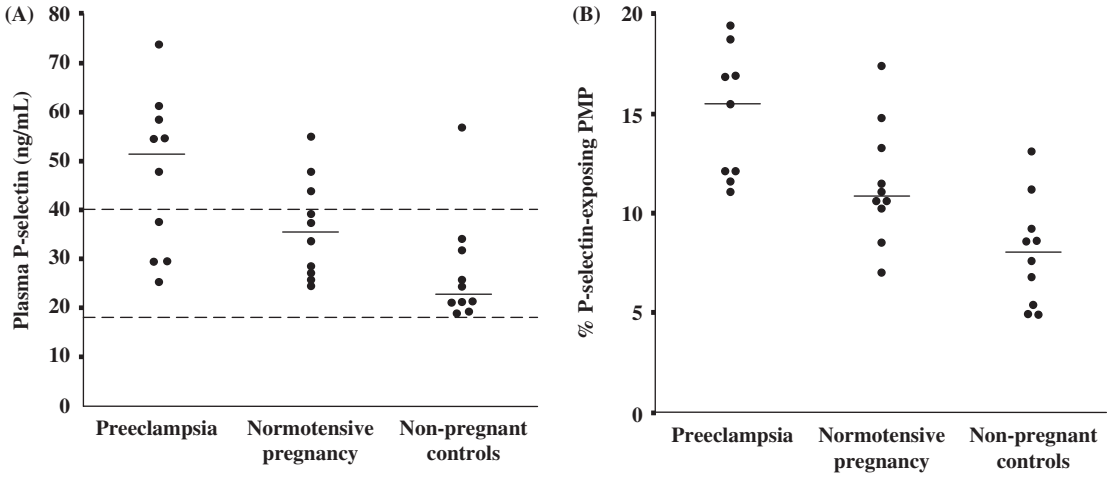

Figure 1. Plasma P-selectin concentration in preeclampsia, normotensive pregnancy and non-pregnant controls. The concentrations of plasma P-selectin (ng/mL) (A) and the percentages of P-selectin-exposing PMP (B) are presented for each individual. The short horizontal lines indicate the median of each separate group. The two dotted lines in graph A represent the reference range as provided by the manufacturer (18–40 ng/mL).

Table II. Number of circulating platelets and (platelet-derived) microparticles.

	Preeclampsia ($n=9$)	Normotensive pregnancy ($n=10$)	Non-pregnant controls ($n=10$)	p	$p^\star$
Platelets ($*10^9$/L)	149 (37–239)	238 (165–396)	304 (155–413)	0.003	0.02
MP ($*10^9$/L)	2.6 (1.3–7.8)	5.1 (1.5–12.8)	6.7 (2.2–16.6)	0.03	0.04
PMP ($*10^9$/L)	2.2 (0.8–7.3)	4.9 (1.4–14.9)	6.6 (1.9–15.6)	0.03	0.03
P-selectin-exposing PMP (%)	15.4 (11.0–19.5)	10.9 (7.0–17.4)	8.0 (4.9–13.1)	0.02	0.001

Data are presented as median (range). p: p-value of difference in means between preeclampsia and normotensive pregnant patients. $p^\star$: p-value of the difference in means between preeclampsia and non-pregnant controls. The p-value of the difference in means between normotensive pregnancy and non-pregnant controls was only significant for the percentage of P-selectin exposing PMP ($p=0.02$). MP: microparticles. PMP: Platelet-derived microparticles.

in plasma can originate from endothelial cells and/or platelets. However, strong correlations between plasma P-selectin concentration and platelet count [14] as well as β-tromboglobulin [15], and a lack of correlation with endothelial activation markers [16], suggest that plasma P-selectin most likely originates from platelets. Increased levels of P-selectin in plasma from patients with platelet consumption disorders, including preeclampsia, provide further evidence for the platelet origin of plasma P-selectin [17]. Our flowcytometry data showed that all MP staining for P-selectin also stained for CD61 (GPIIIa; data not shown). Because MP from human endothelial cells do not expose GPIIIa [18], P-selectin-exposing MP in plasma are likely to originate from platelets and therefore may directly reflect platelet activation.

Overall numbers of PMP were decreased in the preeclamptic group, possibly due to the concurrent decrease in platelet numbers. Another possible explanation is that PMP from preeclamptic patients are attached to leucocytes via P-selectin glycoprotein ligand-1 (PSGL-1) and removed from the circulation. Although the number of PMP was lowest in the preeclamptic group, the fraction of PMP exposing P-selectin was significantly elevated. Thus, subpopulations of PMP in vivo may reflect platelet activation better than overall PMP numbers. These findings emphasize that the composition and function of MP vary, depending on different (patho) physiological circumstances.

Interactions between dysfunctional or damaged endothelium, platelets and MP are likely to contribute to the development of preeclampsia. Recently, Vandendries et al. proposed a model, in which platelets adhere to damaged endothelium, become activated and recruit tissue factor-bearing MP from the blood, leading to coagulation activation and thrombus formation [19]. In their model, P-selectin plays a key role since it not only regulates the initial interactions between leukocytes and the endothelium but also the interactions between activated platelets and leukocytes. It is tempting to speculate that P-selectin-exposing PMP adhere to the (damaged) endothelium in preeclamptic patients, thereby contributing to the recruitment of leukocytes

or their MP, leading to enhanced inflammation and coagulation.

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