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Extensive platelet activation in preeclampsia compared with normal pregnancy: Enhanced expression of cell adhesion molecules

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OBJECTIVES: Platelets play an important role in the pathophysiologic mechanisms of preeclampsia. Our purpose was to investigate by means of flow cytometry to what extent platelets circulate in an activated state during normal pregnancy and whether this activation is more extensive in preeclampsia.

STUDY DESIGN: Platelets in whole blood from 10 preeclamptic third-trimester pregnant women (highest diastolic blood pressure range 100 to 130 mm Hg, proteinuria range 0.59 to 11.5 gm/24 hr) and from 10 normotensive third-trimester pregnant controls were analyzed with the following activation markers: anti-P-selectin (α -granule secretion), anti-CD63 (lysosomal secretion), PAC-1 (monoclonal antibody against fibrinogen receptor conformation of the glycoprotein IIb/IIIa complex), anti-platelet endothelial cell adhesion molecule-1, and annexin-V (a placental protein that binds to negatively charged phospholipids, present on the outside of the platelet plasma membrane after activation). The differences in surface antigen exposure between the two groups were determined by double-label flow cytometry. Flow cytometric data were analyzed in two ways: first, the percentages of activated platelets above a certain threshold compared with a nonpregnant control sample were determined, indicative for activation of a subpopulation of cells, and, second, the mean fluorescence intensities were determined, indicative of the mean surface antigen expression of the total platelet population.

RESULTS: Analysis of the percentage of activated platelets proved most informative. With this analysis an enhanced platelet activation status was present in 4 of 10 normotensive patients and a more extensive platelet activation status in all 10 preeclamptic patients, as indicated by P-selectin ($p = 0.008$) and CD63 ($p = 0.03$) expression. Increased platelet endothelial cell adhesion molecule-1 ($p = 0.005$) expression was also observed in preeclampsia.

CONCLUSIONS: Flow cytometric analysis clearly indicated that platelets circulate in a more extensively activated state during preeclampsia than during normal pregnancy. The increased platelet endothelial cell adhesion molecule-1 expression in preeclamptic patients demonstrates that, besides α -granular and lysosomal release, other hitherto unknown mechanisms are involved. Platelet endothelial cell adhesion molecule-1 appears to be the best marker to distinguish preeclamptic patients from normotensive pregnant women. Only a subpopulation of the platelets appears to be activated. (*Am J Obstet Gynecol* 1997;176:461-9.)

Key words: Preeclampsia, platelet activation, flow cytometry, cell adhesion molecules

Preeclampsia is a complication of pregnancy that may have serious consequences for the mother and the unborn child.¹ It affects approximately 5% of pregnant women and is the most important cause of maternal mortality in the western world. Although the exact cause of preeclampsia remains to be elucidated, evidence

shows that platelets play a substantial role in the pathogenesis of this disease.² Platelets may be activated by damaged endothelium in the systemic circulation or in the placental circulation. Some investigators consider pregnancy as a compensated state of increased platelet consumption, which decompensates in preeclampsia with its extreme in the HELLP syndrome (*hemolysis, elevated liver enzymes, low platelets*).³

Activated platelets have a different exposure of glycoproteins and glycoprotein complexes on their surface compared with nonactivated platelets. Specific monoclonal antibodies have been raised against these glycoproteins, which can serve to detect different states of platelet activation by flow cytometry. Flow cytometric analysis of platelets in whole blood can detect as little as 0.8%

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activated platelets in the circulation.^{4,5} An example of flow cytometric detection of platelet activation in preeclampsia is the finding by Janes and Goodall⁶ of a significantly increased platelet surface expression of CD63, a marker for secretion of platelet lysosomal granules, compared with normotensive pregnancy. In a prospective study Janes et al.⁷ also found an increased expression of CD63 at 28 weeks' gestation in pregnant women before the development of preeclampsia.

Data obtained by flow cytometry to detect cell activation can be analyzed primarily in two ways. First, the expression of activation antigens on the cell surface can be compared with a control sample from a healthy volunteer. The percentage of platelets with an increased antigen expression can then be determined above the threshold, set at for instance 1% to 3% with the control sample. This analysis is indicated if a subpopulation of the cells is extensively activated and the majority are relatively quiescent. Alternatively, the mean fluorescence intensity of cell populations labeled with certain activation antigens can be determined. This mean fluorescence intensity, an indicator of the mean surface exposure of the antigens in the total cell population, can then be compared with that of a healthy control population of nonactivated cells. The latter analysis is the method of choice if some activation of the majority of the cells is anticipated.

We measured platelet activation in preeclamptic and normotensive pregnant women by double-label flow cytometry with the following markers. (1) The first was PAC-1, a monoclonal antibody that binds to the fibrinogen receptor conformation of the GPIIb/IIIa-complex.⁵ (2) The second was anti-P-selectin (PADGEM, GMP-140, CD62P), the α -granular membrane protein that becomes exposed on the plasma membrane after platelet activation associated with α -granular secretion and that is one of the cell adhesion molecules involved in platelet-leukocyte binding.⁸ (3) The third marker was anti-GP53 (CD63), a lysosomal membrane protein that becomes surface exposed on lysosomal secretion.⁹ (4) Anti-platelet endothelial cell adhesion molecule-1 [PECAM-1], CD31 a member of the immunoglobulin superfamily of cell adhesion molecules, was also used. It is expressed on platelets (GPIIa') and leukocytes, and it is present at sites of cell-cell contact on endothelial cells.^{10,11} It is also expressed on the trophoblast.¹² This is of interest because preeclampsia might be considered as an endothelial and trophoblastic cell dysfunction.¹³ Recently PECAM-1 has been localized on three sites of the platelet (i.e., on the α -granular membrane, the open canalicular system, and the surface membrane). After platelet stimulation with thrombin the expression of PECAM-1 on the plasma membrane as detected by immunogold labeling has been reported to increase 44%.¹⁴ (5) The fifth marker was annexin-V, a protein that binds to phosphatidylserine in a Ca^{++} -dependent way. Phosphatidylserine is located on the cytoplasmic side of the plasma membrane of resting platelets and erythrocytes. Platelet activation leads to phosphatidylserine exposure on the extracellular side of the plasma membrane (flip-flop phenomenon).¹⁵ (6) Microparticle analysis was used. During platelet activation small membrane vesicles or microparticles are budded off and can be detected with anti-CD61, a monoclonal antibody that binds to GPIIIa, the integrin β_3 -chain on platelets.⁵ The data were analyzed both as percent activated platelets and as mean fluorescence intensities of the various activation antigens.

Our aim was to investigate whether a panel of activation markers can give an insight into the extent of platelet activation in normal pregnancy and whether this activation is more extensive during preeclampsia. Such a detailed study may enable the selection of marker(s) for a prospective study on the prediction of preeclampsia early in pregnancy. We also evaluated whether the data obtained by flow cytometry indicate that the total platelet population is somewhat activated or whether only a subpopulation of the platelets is activated.

Methods

Patients. Between September 1994 and January 1995, 10 consecutive patients with preeclampsia who were admitted to the obstetric ward of the Academic Medical Center were included. Ten third-trimester pregnant women with uncomplicated pregnancies who visited the outpatient clinic of the same hospital served as control patients. Preeclampsia was defined as a diastolic blood pressure of ≥ 90 mm Hg in combination with proteinuria of ≥ 0.3 gm per 24 hours. The normal pregnant women had no proteinuria with repeated dipstick testing (Albun-test, Boehringer-Mannheim) and remained normotensive throughout pregnancy. Informed consent was given by all patients, and the study was approved by the Ethics Committee at the Amsterdam Medical Center, University of Amsterdam. Patient characteristics are given in Table I.

Materials. PAC-1 was obtained from Dr. S.J. Shattil, Scripps Research Institute, Department of Vascular Biology, LaJolla, California. Anti-CD63 was obtained from Immunotech, Marseilles, France, and fluorescein isothiocyanate-labeled annexin-V from Dr. C. Reutelingsberger, Academic Hospital, Maastricht, The Netherlands. The antibodies anti-GPIb (CLB-MB45), anti-P-selectin (CLB-thromb/6), and anti-PECAM-1 (CLB-ES12F11) were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam. Anti-GPIb was biotinylated by the method of Hnatowitch et al.¹⁷ and the fluorescein isothiocyanate labeling was performed by the method of The and Feltkamp.¹⁸ Heparin was obtained from Organon, Oss,

Table 1. Patient characteristics

	NT	PE	Significance
No. of individuals	10	10	
Age (yr)	33 (28-39)	27 (22-38)	$p = 0.1023$
Amenorrhea at entry study (days)	243 (177-286)	209 (153-256)	$p = 0.0451$
Diastolic blood pressure (mm Hg)			
At first antenatal visit	72 (65-80)	81 (50-85)	$p = 0.46$
At study entry	77 (70-80)	102 (95-125)	$p = 0.0002$
Highest level measured at least twice in pregnancy	76 (70-80)	113 (100-130)	$p = 0.0001$
Whole blood platelet count ($10^9/L$)	222 (138-350)	161 (21-277)	$p = 0.096$
Mean platelet volume (ml)	8.2 (6.7-10.2)	10.5 (8.0-11.3)	$p = 0.549$
β -Thromboglobulin (ng/L)	33 (20-98)	84 (38-183)	$p = 0.026$
Proteinuria ($gm \cdot 24 hr^{-1}$)	*	3.7 (0.6-11.5)	$p = 0.0001$
Birth weight(gm)	3072 (1980-4170)	1528 (250-3020)	$p = 0.012$
Birth weight percentiles ¹⁶	47 (5-90)	10 (2-50)	$p = 0.0022$

Values are presented as mean, with range in parentheses. Normal β -thromboglobulin plasma levels ≤ 35 ng/L. NT, Normotensive pregnant women; PE, preeclamptic patients.

*Negative on dipstick testing (Albim-test, Boehringer Mannheim).

The Netherlands, and phycoerythrin-conjugated streptavidin from Dakopatts, Glostrup, Denmark. Only monoclonal antibodies were used.

Preparation of blood samples. Blood was obtained from an antecubital vein through a butterfly needle (1.0 mm) with either no or a light tourniquet. The first 4 ml of blood was discarded. Then 3.6 ml of blood was collected with 0.4 ml of PECT anticoagulant, which consists of (1) 270 mmol/L sodium ethylenediaminetetraacetic acid, (2) 30 mmol/L theophylline diluted in phosphate-buffered saline solution, and (3) 1.9 mmol/L sodium carbonate and 282 nmol/L prostaglandin E_1 (1:1:1 diluted) for β -thromboglobulin determination. Then a 2.7 ml sample of blood was collected in 0.3 ml of 3.2% sodium citrate. Subsequently, a 2 ml sample of blood was collected in a tube containing 8 μ l heparin, 5000 IE/ml. Within 3 minutes 5 μ l aliquots of the citrated blood were added to tubes containing 35 μ l HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) buffer (137 mmol/L sodium chloride, 2.7 mmol/L potassium chloride, 1.0 mmol/L magnesium chloride, 5.6 mmol/L glucose, 20 mmol/L HEPES, 1 mg/ml albumin, 3.3 mmol/L sodium phosphate, pH 7.4), 5 μ l anti-GPIb (1.5 μ g/ml final concentration), and 5 μ l of a fluorescein isothiocyanate-labeled second antibody. For detection of activation with annexin V, 5 μ l of the heparin-anticoagulated blood was added to tubes containing 35 μ l of the annexin V HEPES buffer and 5 μ l of anti-GPIb (the same HEPES buffer but including 1.8 mmol/L calcium chloride instead of 3.3 mmol/L sodium phosphate). Final concentrations were 30 μ g/ml PAC-1, 10 μ g/ml anti-P-selectin, 5 μ g/ml anti-CD63, 3 μ g/ml annexin V, 2.5 μ g/ml anti-CD61, and 1 μ g/ml anti-PECAM-1. In preliminary in vitro experiments these concentrations yielded optimal detection of the platelet antigens. Of circulating cells, GPIb is solely confined to platelets, so anti-GPIb monoclonal antibodies can be

used to select platelets and platelet-derived material in the flow cytometric analyses.

After careful mixing and 15 minutes of incubation at ambient temperature in the dark, 5 μ l of 10-fold diluted phycoerythrin-conjugated streptavidin was added. Incubation continued for another 15 minutes, and finally the samples, except for the annexin V samples, were diluted by the addition of 2.0 ml of HEPES buffer. Five microliters of the annexin V incubation mixture was added to 250 μ l of annexin V HEPES buffer supplemented with 3 μ g/ml annexin V.

With each study of a preeclamptic patient or a normal pregnant woman, blood was simultaneously collected from a healthy nonpregnant control subject and processed identically. Those samples served to set thresholds for the platelet activation in the flow cytometric analysis at approximately 1% to 3%.

Flow cytometric analysis. Double-label flow cytometry was performed as described by Shattil et al.⁴ Within 2 hours after collection the samples were analyzed in a FACScan flow cytometer with PC-lysis software (Becton-Dickinson, San Jose, Calif.). Forward light scatter and sideward light scatter were set at logarithmic gain. Platelet material was identified by analyzing the biotinylated GPIb content of particles at 585 nm. The platelets (R2, 70% to 75% of all platelet-derived particles) were distinguished from microparticles (R1) and platelet-leukocyte complexes (R3) by measuring their size on gating in the forward scatter versus side scatter mode (Fig. 1).

The surface expression of activation markers on a population of 5000 platelets was determined by the fluorescence intensity of the fluorescein isothiocyanate-labeled antibodies at 515 nm. For the data analysis to determine the percent activated platelets, the threshold for platelet activation was set at approximately 1% to 3% fluorescence-positive platelets in the healthy nonpregnant control samples. Subsequently, the percentages of

Fig. 1. Distribution of particles identified with platelet-specific anti-GPIb in healthy control. Side scatter (SSC) is plotted versus forward scatter (FSC). R1 contains platelet-derived microparticles, R2 actual platelet population, and R3 platelet-platelet or platelet-leukocyte complexes.

platelets from preeclamptic patients or normotensive pregnant women with fluorescein isothiocyanate fluorescence above these thresholds were determined. The percentage of positive platelets in the nonpregnant control sample was then subtracted to obtain the final result as percentage activated platelets. An example of such a determination is given in Fig. 2. In the second data analysis the mean fluorescence intensities, measured as mean channel number as an indicator of mean surface antigen expression of the total platelet population, for each patient and the controls were obtained by analyzing histograms from each R2 region. Microparticles were also identified with logarithmic gain settings for forward and sideward light scatter. The forward light scatter setting was E01, and the FI-threshold was set just above the autofluorescence (variable per patient and per control subject).

In preliminary experiments all antibodies used were tested by platelet stimulation with thrombin. Blood from healthy nonpregnant volunteers was used for these experiments. Maximal stimulation of platelets, isolated by gel filtration, with thrombin (0.1 U/ml) showed an increase from 2% activated platelets to 41% for P-selectin, 86% for CD63, 90% for PAC-1, and only 8% for PECAM-1.

β -Thromboglobulin determination. Blood in PECT anticoagulant was centrifuged at 2690g for 60 minutes at 4° C to obtain platelet-free plasma. β -Thromboglobulin was measured by enzyme-linked immunosorbent assay (Asserachrom, Diagnostica Stago, Asnières, France).

Statistics. Differences between groups were analyzed with the Mann-Whitney *U* test.

Results

Patient characteristics. All patients had severe preeclampsia, as evidenced by the range of the maximal diastolic blood pressures, extent of proteinuria, and birth weights (Table I). One of the patients had HELLP syndrome. The normotensive pregnant and preeclamptic patients did not differ in blood pressure at the first antenatal visit, nor was there a difference in maternal or gestational age. As expected, the groups differed significantly in blood pressure at study entry ($p = 0.0002$), in maximal blood pressure throughout pregnancy ($p = 0.0001$), in proteinuria ($p = 0.0001$), and in birth weight percentiles (Table I).

Platelet count and mean platelet volume. Platelet count and mean platelet volume did not differ significantly between the normotensive and preeclamptic patient groups (Table I).

β -Thromboglobulin levels. β -Thromboglobulin levels were increased in the preeclamptic group compared with the normotensive group (Table I).

Platelet activation analyses. The results of the data analysis as percent platelet activation are given in Table II. The median percentages of activated platelets as assessed by the surface expression of P-selectin ($p = 0.008$), CD63 ($p = 0.028$) or PECAM-1 ($p = 0.005$) were significantly higher in the preeclamptic patients than in the normotensive pregnant women. Logistic regression analysis showed that PECAM-1 is the best marker to distinguish preeclamptic patients from normotensive pregnant women. In contrast, annexin V binding, indicating surface exposure of negatively charged lipids, and PAC-1 binding to indicate fibrinogen receptor availability were not significantly different between these groups. Nor did the percentages of microparticles differ between the normotensive pregnant women and the preeclamptic patient groups. However, interestingly, the one preeclamptic patient with excessive presence of microparticles (42%) also had the most severe thrombocytopenia (22×10^9 platelets/L).

A more critical appraisal of the platelet activation analyzed as percent activated cells indicates that the results are not uniform within the two patient groups. With all activation markers the percentages of activated platelets vary from below the level of the nonpregnant control to increased levels (Fig. 3). In the data analysis to determine the percent activated platelets in a patient compared with the sample from a healthy control, negative percentages were frequently encountered. This is caused by the variable biologic expression among the healthy controls. Because of this variability, it is also arbitrary to consider a certain percentage to be truly indicative of the presence of an enhanced platelet activation status in a patient. In the current study we considered a surface antigen expression $>3\%$ above the level of the healthy control indicative of platelet activa-

Fig. 2. Determination of activation status of platelet population. Analysis of PECAM-1 expression in patient is presented. Platelets (R2, as in Fig. 1) were selected and compared with the control sample from healthy volunteer. With control sample, threshold for antigen analysis was arbitrarily set at 1% to 3%. In this example 2.9% PECAM-1-positive platelets were arbitrarily set in control sample (left) and then 25.8% was present in sample taken from this patient (right). Resulting difference in activation status is then 22.9%.

Table II. Analysis of percent activated platelets

	NT	PE	Significance
P-selectin	0.0 (-1.0-5.8)	4.0 (1.0-20.2)	$p = 0.008$
CD63	0.1 (-2.0-6.6)	8.0 (-1.6-38.6)	$p = 0.03$
PAC-1	1.0 (-1.4-5.2)	0.5 (-1.5-3.6)	$p = 0.56$
PECAM-1	1.4 (-0.7-10.3)	10.2 (1.5-23.6)	$p = 0.005$
Annexin V	0.6 (-0.5-45.9)	0.5 (-0.2-2.7)	$p = 0.49$
Microparticles (%)	3.4 (-6.2-15.7)	3.4 (-7.7-42.4)	$p = 0.73$

Percent platelet activation indicated as median and range. Differences in percent platelet activation were established and analyzed with the Mann-Whitney *U* test. NT, Normotensive pregnant women; PE, preeclamptic patients.

tion status. At a level $\geq 3\%$ of platelet activation four normotensive pregnant women demonstrated evidence of some enhanced platelet activation status compared with the nonpregnant women serving as controls, as indicated by one or more of the markers P-selectin, CD63, and PECAM-1. At the same 3% decision limit, all 10 preeclamptic patients demonstrated evidence of an enhanced platelet activation status.

The results of the second data analysis, to determine the mean fluorescence intensities as mean antigen expression of the whole platelet population, are presented in Table III. Median values of the mean fluorescence intensities for all markers were slightly, but not significantly, increased in the preeclamptic patients compared with the normotensive healthy pregnant women. The mean fluorescence data of each individual are presented in Fig. 4. The mean fluorescence data of all markers used in this study had a large range in the healthy nonpregnant control women. The median values of the mean fluorescence intensities of all markers used did not differ substantially among healthy nonpregnant control women, healthy pregnant women, and preeclamptic patients. Compared with the 97.5th percentile of the mean fluorescence intensity of the healthy nonpregnant

control women, the individual data in the healthy pregnant women also did not indicate extensive increased mean fluorescence intensities. With PECAM-1, five of the preeclamptic patients had a mean fluorescence intensity > 97.5 th percentile of the nonpregnant healthy control women. However, with annexin V, P-selectin, PAC-1, and CD63 only one, four, one, and three preeclamptic patients had an increased antigen exposure, respectively.

Within the patient group we correlated the birth weight, extent of proteinuria, and maximal diastolic blood pressure with the percent activated platelets with use of P-selectin, CD63, and PECAM-1 as the positive markers. We did indeed observe a positive correlation between the extent of proteinuria and the CD63 expression (Spearman $r = 0.87$, $p < 0.001$) but not with all other correlations ($r < 0.5$). Also, the patient with the HELLP syndrome had the highest CD63 expression and extent of proteinuria (but only intermediate P-selectin and PECAM-1 expression).

Comment

Analysis of the flow cytometric data as mean fluorescence intensities (i.e., mean surface expression of the total platelet population) provided only a minor indica-

Fig. 3. Individual percentages of platelet activation and median values (*horizontal solid line*) for preeclamptic patients and normotensive pregnant women are represented. *Left*, Significant platelet activation markers; *right*, nonsignificant platelet activation markers. **A**, PECAM-1. **B**, P-selectin. **C**, CD63. **D**, Annexin V. **E**, PAC-1. **F**, Microparticles. *Solid circles*, Patients with preeclampsia; *open circles*, normotensive pregnant women.

tion of an enhanced activation status of these cells in preeclamptic patients and in healthy pregnant women. Median values of the mean fluorescence intensities were not statistically significantly increased in preeclamptic patients compared with normotensive healthy pregnant women. The number of preeclamptic patients with a mean fluorescence intensity >97.5th percentile of the range in healthy nonpregnant controls was limited to only five for PECAM-1 and, respectively, four and three with P-selectin and CD63. In contrast, the increased expression of the antigens P-selectin, CD63, and PECAM-1 on the platelet surface in some normotensive pregnant women and more extensive platelet activation in the preeclamptic women was demonstrated in the analysis of the percentages of platelet activation. These findings indicate most likely that in preeclampsia, a subpopulation of platelets is activated, rather than activation of the whole platelet population. This subpopulation is only modestly activated if the surface expression of

P-selectin, PAC-1, or CD63 induced by thrombin *in vitro* with the data obtained in these patients are compared (unpublished results). Possibly more extensive platelet activation occurs in preeclampsia, but such platelets are then rapidly cleared from the circulation.

We did not observe a clear correlation between the severity of the preeclampsia and the extent of platelet activation. Only the increased expression of CD63 and the degree of proteinuria was substantially and significantly correlated ($r = 0.87$, $p < 0.001$) and both parameters were highest in the patient with HELLP syndrome.

PECAM-1 proved to be the best marker to distinguish between preeclamptic patients and normotensive pregnant women. P-selectin and CD63 are established indicators of secretory phases of the α -granular and lysosomal constituents during platelet activation.^{8,9} For PECAM-1 the association between platelet activation and increased surface exposure is debatable. So far, PECAM-1 expression on the platelet has not been studied in clinical

Table III. Analysis of mean fluorescence intensities

	<i>NT</i>	<i>PE</i>	<i>Significance</i>
P-selectin	10.3 (7.5-11.5)	11.3 (8.0-15.3)	$p = 0.09$
CD63	7.2 (6.7-10.0)	8.2 (6.2-21.5)	$p = 0.29$
PAC-1	15.8 (5.3-42.5)	18.7 (3.6-124.3)	$p = 0.2$
PECAM-1	64.3 (50.5-73.6)	70.7 (46.6-112.1)	$p = 0.06$
Annexin V	27.0 (20.4-33.3)	27.1 (14.6-58.2)	$p = 0.3$

Mean fluorescence intensities for each normotensive (*NT*) or preeclamptic (*PE*) pregnant woman were first established for each marker. Median values of these data within groups are provided, with range of those values in parentheses. Statistical significance was analyzed with Mann-Whitney *U* test.

conditions. After thrombin stimulation of platelets *in vitro* the PECAM-1 expression as detected by immunogold labeling has been reported to increase 44% on the platelet surface and was presumed to indicate α -granular release.¹⁴ However, in preliminary experiments to investigate the maximal stimulation of gel-filtered platelets with thrombin (0.1 U/ml) we found an increase in PECAM-1 expression on a subpopulation of only 8% of all platelets by means of flow cytometry, whereas under these conditions the surface expression of CD63 and P-selectin was increased to 86% and 41%, respectively (unpublished results). Thus the increased PECAM-1 expression on the platelet surface in preeclamptic patients may not indicate α -granular release. PECAM-1 has also been reported to be localized at the open canalicular system.¹⁴ The PECAM-1 expression of the preeclamptic patients, analyzed by percent platelet activation, was significantly correlated with the forward scatter ($r = 0.50$, $p = 0.0001$), which is a determinant of platelet size. It is known that platelet size increases in pregnancy and even more in pregnancies complicated with preeclampsia,² so the increased PECAM-1 expression could be due to an increased availability of PECAM-1 in the open canalicular system to the antibody. The channels of this system have indeed been reported to evaginate after platelet activation and shape change.¹⁹ Alternatively, uptake of soluble PECAM-1 from plasma could be considered. We did measure plasma concentrations of soluble PECAM-1 with an enzyme-linked immunosorbent assay. The plasma concentrations were not increased in preeclamptic patients compared with normotensive pregnant women (preeclamptic patients mean 46 ng/ml and normotensive pregnant women mean 44 ng/ml, $p = 0.92$). Binding of soluble PECAM-1 to the surface of the platelet therefore seems unlikely to be the cause of the enhanced platelet surface exposure in preeclampsia. However, these findings do not completely exclude the possibility that some shedding of soluble PECAM-1 from activated endothelial cells occurs, which is then obscured in the circulation by cellular scavenging of the soluble PECAM-1.

Whatever the mechanism, preeclampsia is the first clinical disorder with an established increased PECAM-1

exposure on the platelet surface. The increased expression of P-selectin, CD63, and possibly PECAM-1, together with the increased plasma β -thromboglobulin concentrations found in the preeclamptic patients, confirm the previously reported increased secretion of the content of α - and lysosomal granula in preeclamptic women.²⁰

PECAM-1 and P-selectin are cell adhesion molecules. The known functions of these molecules may indicate their importance in leukocyte and endothelial cell activation in preeclampsia. Activation of leukocytes and endothelial cells has been clearly established in preeclampsia.^{13, 21} For instance, activated neutrophils are known to release oxygen free radicals and an increased production of superoxides is present in preeclampsia.²² These radicals damage vascular integrity and endothelial cell function and cause platelet activation.¹³ How can P-selectin and PECAM-1 expression on the platelet contribute to endothelial cell and leukocyte activation? P-selectin is a cell-adhesion molecule present in the membranes of α -granules of platelets and the Weibel-Palade bodies of the endothelial cell.²³ On stimulation of these cells (e.g., by thrombin or oxygen radicals, respectively) the content of these granules is secreted to the extracellular environment of the cell by fusion of the granular membrane with the outer cell membrane. This fusion causes the exposure of P-selectin to the outside of the activated cell. On the endothelial cell surface P-selectin is involved in the reversible Ca^{++} -dependent binding of leukocytes, which causes the so-called rolling of the leukocyte on the endothelial cell lining of the vessel wall.¹¹ On the activated platelet P-selectin mediates the binding to leukocytes, including monocytes, neutrophils, and various subsets of T lymphocytes. On the monocyte the ligand involved in the binding to P-selectin has been identified.²⁴ The platelet-monocyte binding causes the increased expression of tissue factor, a major initiator of the coagulation system, on the monocyte. Thus increased P-selectin expression on the platelet facilitates platelet-leukocyte and possibly platelet-endothelial cell interactions.

PECAM-1 is concentrated at the junctions of vascular endothelial cells and is also expressed on platelets, monocytes, neutrophils, a subset of T cells, and the

Fig. 4. Individual values of mean fluorescence intensities, measured as mean channel numbers, are represented. **A**, PECAM-1. **B**, P-selectin. **C**, CD63. **D**, Annexin V. **E**, PAC-1. The 2.5 and 97.5 percentiles (vertical solid line) and median values (horizontal solid line) for preeclamptic patients ($n = 10$) (solid circles), normotensive pregnant women ($n = 10$) (open circles), and nonpregnant healthy control women ($n = 20$) (stippled circles) are represented as well.

intercellular endothelial junctions of the human term placenta.^{10, 25} PECAM-1 serves both as the receptor and as its ligand in cell-cell interactions. PECAM-1 on endothelial cells plays an important role in the transendothelial migration of leukocytes. PECAM-1 expression on monocytes and neutrophils can result in increased adhesive properties of these cells. Possibly, increased exposure of PECAM-1 on the platelet surface in preeclampsia promotes its binding to the interendothelial junctions of the (micro)vasculature of the placental bed, causing the characteristic pathologic changes through platelet and leukocyte activation, thus causing endothelial damage in the maternal systemic circulation.²⁵

PAC-1 expression on the platelet surface was not increased in this study. However, PAC-1 only binds to the active conformation of the fibrinogen receptor when it is not occupied with fibrinogen. In preeclampsia it is likely that the fibrinogen receptor is indeed occupied with fibrinogen, because fibrinogen is readily available in the circulation. Studies with antibodies that bind to the

GPIIb/IIIa-fibrinogen complex (i.e., against ligand-induced binding sites and receptor-induced binding sites) will clarify this issue.²⁶ Annexin V expression was also not elevated in preeclampsia. Evidently there is no exposure of phosphatidylserine on the outer surface of the plasma membrane of the circulating platelets in preeclampsia. Rapid clearance of such platelets from the circulation might explain this. Finally, the percentages of microparticles did not differ between the two groups. Only one preeclamptic patient with the most severe thrombocytopenia ($22 \cdot 10^9$ cells/L) showed a considerably increased percentage of microparticles (42%). Microparticles can be readily demonstrated in *in vitro* experiments, but so far their presence in clinical disorders has only been established in patients undergoing cardiopulmonary bypass surgery.

In conclusion, this study demonstrates that platelets circulate in an enhanced activation state in some normotensive pregnant women. The platelet activation status is more enhanced and present in all preeclamptic women

investigated, as evidenced by the increased expression of P-selectin and CD63. The increased PECAM-1 expression in preeclamptic patients demonstrates that, besides α -granular release, other hitherto unknown mechanisms of increased surface antigen exposure are also involved. P-selectin, CD63, and PECAM-1 are potential markers to establish platelet activation early in pregnancy as an indicator for oncoming preeclampsia. Finally, the two separate analyses of the flow cytometric data indicate that in general only a subpopulation of the platelets has an enhanced activation status in the preeclamptic patient.

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