Blood Coagulation, Fibrinolysis and Cellular Haemostasis

Circulating microparticles: a marker of procoagulant state in normal pregnancy and pregnancy complicated by preeclampsia or intrauterine growth restriction

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Summary
In the present study, we explored the microparticles involved in the control of hemostatic equilibrium, i.e. microparticles originating from platelet, endothelial cells and total MP defined as annexin V positive microparticles. Our aim was to analyze the level and procoagulant activity of these microparticles in normal pregnancy and pregnancies complicated with preeclampsia or isolated intrauterine growth restriction. We reported increased levels of platelet and endothelial microparticles in normal pregnancy compared to non pregnant healthy women. Number of annexin V microparticles was significantly increased together with their procoagulant activity. In pathological pregnancies, significant reduction in platelet microparticle number was found in preeclampsia. The procoagulant activity generated by the total annexin V MP was unchanged, suggesting that the microparticles remaining in the circulation were procoagulant. This study evidenced that microparticles constitute a cellular marker of a proinflammatory and procoagulant responses in normal pregnancy. In pregnancies with vascular complications, circulating MP with procoagulant potential may be part of the exacerbation of these responses.

Keywords
Pregnancy, microparticles, preeclampsia, intrauterine growth restriction

Introduction
Normal pregnancy is considered to be an inflammatory and hypercoagulable state with increased incidence of thromboembolic events (1). During normal pregnancy, changes in the haemostatic mechanism involve increased levels of coagulation factors and/or decreased levels of anticoagulant protein such as antithrombin and protein S as well as enhanced thrombin generation and decreased fibrinolytic activity (2, 3). Preeclampsia (PE) and intrauterine growth restriction (IUGR) are vascular complications that occur in 5 to 7% of pregnancies. The pathophysiology of PE and IUGR remains unclear although they...
Materials and methods

58 pregnant women who gave informed consent were recruited for the study. 24 women had PE defined as persistent diastolic blood pressure above 90 mmHg, systolic blood pressure above 140 mmHg associated with proteinuria greater than 300 mg per 24 h. These patients were normotensive before 20 weeks of gestation. 18 women exhibited intra-uterine growth restriction defined by ultrasonographic measurement < 5th percentile for gestational age and confirmed by child weight at birth according to Brenner et al. (20). The probable vascular origin of IUGR was shown by reduced placental vessel perfusion assessed by Doppler’s waves flow velocimetry. Fetus presenting growth restriction had normal echographic examination. The exclusion criteria for women whose pregnancies were complicated by IUGR were the presence of congenital malformations or chromosomal abnormalities in the fetus, recent cytomegalovirus infection, drug or alcohol abuse during pregnancy and clinical signs of maternal PE as defined above. The ratio between birth weight and normal expected birth weight was evaluated according to Leroy-Lefort tables and used as a retrospective severity criterion. A group of 15 age-and gestation-matched women were selected as healthy pregnant subjects. These healthy women were primigravidae with no history of medical illness, attending the routine antenatal clinics. They presented an uneventful pregnancy at the day of inclusion and no vascular complications until the end of pregnancy. Exclusion criteria for healthy pregnant women were arterial blood pressure upper 140/90 mmHg and proteinuria > 300 mg/24 h.

19 age-matched healthy non pregnant women were selected as a control group. None of the patients received any anti-platelet treatment. The presence of antiphospholipid antibodies was an exclusion criteria for all groups including normal pregnancy.

MP harvesting and preparation

Whole blood was drawn on sodium citrated tube (0.129 M) and treated within 2 h. Briefly, samples were centrifuged at 1500 g during 15 min, followed by a 2 min centrifugation at 13,000 g. 30 μl of the plasma were then incubated with specific monoclonal antibodies directed against endothelial and platelet antigens (respectively: anti-αvβ3: FITC-CD51, clone AMF7 and anti-GPIIbIIIa: PE-CD41, clone P2, both from Beckmann Coulter Immunotech, Marseille, France). In order to define the background noise of the cytometric analysis, plasmas were also labelled with corresponding isotype-controls (FITC-IgG1 and PE-IgG1, from Beckmann Coulter Immunotech, Marseille, France). To enumerate total MP, 30 μl of plasma were incubated with FITC-annexin V that binds to the phosphatidylserine present at the MP surface. After 30 min of incubation, samples were diluted in PBS or binding buffer and internal standard (Flowcount beads, Beckmann Coulter,) was added to express counts of MP as absolute numbers. Samples were then analysed...
by cytometry as previously described (16). Briefly, on a LogFS-LogSS dotplot, the MP upper size limit was defined using 1 μm beads, and the MP gate was drawn around the population. The lower limit of the gate excludes the first channel that contains the electronic background noise of the machine. Only the events included within this MP gate were analysed for their fluorescence on a LogFL-LogSS dotplot. Results were expressed as number of MP/μl of plasma.

**Procoagulant activity**

The microparticle procoagulant activity was determined, using a prothrombinase assay after capture on a microtitration plate coated with annexin V, as previously described (21). The blood clotting factor concentrations were determined to ensure that phosphatidylserine is the rate-limiting parameter of the reaction and results were expressed as nanomolar phosphatidylserine equivalent by reference to a standard curve constructed using liposomes of defined composition.

**Statistical analysis**

Since data were not normally distributed, results were expressed as a median and range and statistical analysis was performed with GraphPad statistical software. In a first step, to investigate whether microparticles parameters were modified during normal course of pregnancy, we compared healthy pregnant women and healthy non pregnant women using non-parametric Mann-Whitney test. In a second step, we aimed to evaluate microparticles parameters in pregnancy complicated with preeclampsia or isolated IUGR compared to normal pregnancy. For this purpose, we used Kruskall-Wallis test followed by post-hoc test. p < 0.05 was considered significant. Correlation between microparticle parameters and markers of severity of PE or IUGR were evaluated with a Spearman test.

**Results**

**Characteristics of patients**

Clinical and biological findings from patients and controls are summarised in Table 1. The different groups were comparable with respect to age. There was no significant difference in gestational age at sampling between pregnant groups. Uricemia and fibronectin were significantly higher in PE group compared to healthy pregnant women, whereas only fibronectin was

<table>
<thead>
<tr>
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<th>PE± IUGR</th>
<th>Isolated IUGR</th>
<th>Normal pregnancy</th>
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<tbody>
<tr>
<td></td>
<td>N=24</td>
<td>N=18</td>
<td>N=15</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>160 [140-200] *</td>
<td>120 [90-155] §</td>
<td>110 [100-130]</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>90 [70-110] *</td>
<td>70 [50-95] §</td>
<td>80 [60-95]</td>
</tr>
<tr>
<td>Proteinuria (g/24h)</td>
<td>1 [0.3-5]*</td>
<td>0 [0-1]§</td>
<td>0 [0-0.39]</td>
</tr>
<tr>
<td>Fibronectin (ng/ml)</td>
<td>0.58 [0.4-0.91] *</td>
<td>0.48 [0.24-0.77]</td>
<td>0.39 [0.33-0.5]</td>
</tr>
<tr>
<td>Uricemia (μmol/ml)</td>
<td>319 [208-478]*</td>
<td>260 [151-383]§</td>
<td>227 [153-304]</td>
</tr>
<tr>
<td>Platelet count (G/l)</td>
<td>230 [64-424]</td>
<td>224 [139-583]</td>
<td>200 [100-339]</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)</td>
<td>31 [24-38]*</td>
<td>32 [24-39]*</td>
<td>41 [36-41]</td>
</tr>
<tr>
<td>Birth Weight (g)</td>
<td>1350 [450-4325] *</td>
<td>1225 [430-2750] *</td>
<td>3448 [2330-4110]</td>
</tr>
<tr>
<td>Expected birth weight / birth weight</td>
<td>0.71 [0.58-1.2] *</td>
<td>0.6 [0.37-0.86] *</td>
<td>0.99 [0.82-1.16]</td>
</tr>
<tr>
<td>Number of patients with birthweight below 5th centile</td>
<td>17</td>
<td>18</td>
<td>none</td>
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*D p <0.05 compared with normal pregnancy, § p <0.05 compared with pre-eclampsia group.*
elevated in IUGR group. As expected the birth weight, the ratio between expected birth weight and birth weight and gestational age at delivery were significantly lower in PE and IUGR compared to normal pregnancy. A birth weight below the 5th centile for gestation was observed in 70.8% of patients with preeclampsia.

**Microparticles in normal pregnancy**

Microparticles of endothelial (EMP) and platelet (PMP) origin were enumerated by flow cytometry using specific monoclonal antibodies directed against the integrin αvβ3 and the glycoprotein IIbIIIa respectively. As shown in Table 2, the plasma levels of EMP and PMP were significantly increased in healthy pregnant women compared to non pregnant controls. Since at present, no methodology is available to evaluate the procoagulant activity of the MP specifically released by a cell type, the whole population of microparticles positive for annexin V was considered for procoagulant activity measurement. Both the number and procoagulant activity of these annexin V positive-MP were significantly increased in pregnant controls compared to non pregnant controls.

**Microparticles in complicated pregnancy**

The levels of circulating MP were then analyzed in PE and isolated IUGR compared to normal pregnancy. The levels of PMP significantly differed between groups (overall p value 0.026). In pathological pregnancies, we observed a decrease of PMP number which reach statistical significance only for PE (37.5 [8-451] and 90 [5-1202] for PE and isolated IUGR respectively compared to 193 [14-773] in normal pregnancy) (Fig. 1A). No significant decrease was observed for total annexin V positive microparticles (260 [10-446] and 182 [83-650] for PE and IUGR groups, compared to 429 [260-1598] for pregnant controls (Fig. 2A). Their procoagulant activity was also not decreased (Fig. 2B). No significant difference in the numeration of MP derived from endothelial cells was observed in PE (9 [2-32]) nor in IUGR (12 [1-58]) compared to healthy pregnant women (13 [1-32]) (Fig. 1B).

**Correlation studies**

We investigated whether there was a correlation between the levels or procoagulant activity of circulating MP and criteria of disease severity. In the PE group, neither levels of platelet, endothelial and total MP nor MP procoagulant activity were correlated with proteinuria or blood pressure. In the IUGR group, microparticles parameters did not correlate with the ratio between birth weight and expected birth weight. In addition, we found no significant correlation between MP parameters and uric acid or fibronectin levels.

**Discussion**

This study is the first to demonstrate that normal pregnancy is associated with increased numbers and procoagulant activity of cell-derived MP. Unexpectedly, compared to normal pregnancy, pathological pregnancies did not result to higher levels of microparticles but were associated to lower number of platelet and total annexin V MP. In addition, these decreased numbers of MP were not associated with a concomitant decrease of their procoagulant activity.

Exploration of circulating microparticles has become a valuable marker for detection of in vivo cell activation. The presence of increased numbers of platelet and endothelial MP in normal pregnancy reflects an ongoing process of activation affecting these cells. Inflammatory stimuli are known to be the main inducers of membrane vesiculation and we have shown that cultured endothelial cells released microparticles upon stimulation with TNFα (16). Interestingly, recent studies have evidenced high plasma levels of TNFα and IL-6 in healthy pregnant women (22). We can therefore suggest that, in normal course of pregnancy, these proinflammatory cytokines, are involved in cell vesiculation. Since procoagulant activity of microparticles results from the external exposure of anionic phospholipids which support prothrombinase complex assembly, we were interested in the level of annexinV positive-microparticles. We demonstrated that, together with increased number, the circulating microparticles associated to normal pregnancy carried enhanced procoagulant

**Table 2:** Comparison of microparticle numeration and procoagulant activity in non pregnant women and control pregnant women. Results were given as number of MP/μl of plasma. Values are represented as median [range]. Difference between groups was analyzed by Mann-Whitney U test.

<table>
<thead>
<tr>
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<th>Non pregnant controls N = 19</th>
<th>Healthy pregnant women N = 15</th>
<th>Statistics</th>
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<tr>
<td>Endothelial MP (μl)</td>
<td>7 [1-21]</td>
<td>13 [1-32]</td>
<td>p=0.04</td>
</tr>
<tr>
<td>Platelet MP (μl)</td>
<td>39 [17-674]</td>
<td>193 [14-773]</td>
<td>p = 0.0006</td>
</tr>
<tr>
<td>Total Annexin V positive MP (μl)</td>
<td>122 [18-447]</td>
<td>429 [260-1598]</td>
<td>p = 0.0005</td>
</tr>
<tr>
<td>Procoagulant activity Eq nM PS</td>
<td>8 [1.8-11.6]</td>
<td>11.9[3.9-25]</td>
<td>p=0.018</td>
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activity compared to those of non pregnant healthy controls. Consequently, they may contribute to the hypercoagulable state of normal pregnancy which, until now, was only documented by changes in plasma molecules involved in the clotting system such as fibrinogen, factor VIII, protein S or antithrombin. High level of circulating MP can be part of the generalised procoagulant and inflammatory reaction accompanying maternal adaptation to pregnancy. In PE and IUGR, the physiological inflammation of pregnancy is thought to decompensate because of excessive stimuli from poorly perfused placenta and/or maternal susceptibility factor. Findings from animal models have shown that preeclampsia can be induced in pregnant mice by low dose of endotoxin, whereas non pregnant mice are unaffected by the same inflammatory stimulus (23). These data have led to the concept that vascular obstetrical complications like PE or IUGR arise from exacerbation of the inflammatory response to pregnancy (24, 25). Surprisingly, our results demonstrated that women with PE displayed lower numbers of circulating MP than healthy pregnant women. This decrease mainly concerned platelet-microparticles and is consistent with the observation of Harlow et al. (26). Similar decrease in MP number has been previously reported in pathological situations associated with severe proinflammatory conditions, like multiple organ dysfunction syndrome and sepsis (27). MP decrease is unrelated to the number of circulating cells since platelet or leukocyte count were unchanged in pathological pregnancies. One possible cause of platelet microparticle removal from peripheral circulation may be their consumption by excessive clotting reactions occurring in the pathological placental beds. Consistently fibrin deposits have been evidenced in placental vasculature of women with PE or IUGR (28) and platelet microparticles have been shown to adhere to fibrin (29). In addition, phosphatidylserine vesicles injected to into pregnant mice concentrated in placental tissue, induced thrombin generation in the placental circulation and led to reduced birthweight (30). In addition, MP could be cleared from the plasma by adhering to blood or vascular cells. Indeed, MP derived from platelet or polymorphonuclear cells can bind to monocytes or endothelial cells respectively (15, 31). In PE and
IUGR, MP interaction with leukocytes or endothelial cells may be promoted by the strong activation of these cells largely documented by metabolic features, changes in leukocyte phenotype (32) and soluble endothelial markers. Furthermore, recently MP have been shown to induce biological responses in cells they interact with (33, 34). Consequently, recently MP have been shown to induce biological responses in cells they interact with (33, 34). Consequently, recently MP have been shown to induce biological responses in cells they interact with (33, 34). Consequently, recently MP have been shown to induce biological responses in cells they interact with (33, 34). Consequently, recently MP have been shown to induce biological responses in cells they interact with (33, 34).

Compatible with this deleterious effect of microparticles, we have recently demonstrated that EMP can induce monocyte procoagulant activity (35). The lower decrease of MP observed in IUGR suggests that both placental dysfunction and cellular adhesive interactions may potentially participate in microparticles clearance from circulation but in a lesser extent. This is consistent with the usual restriction of vascular abnormalities to the foeto-placental unit in IUGR. Despite a trend toward lower levels of annexin V positive-MP in PE and IUGR, the global procoagulant activity generated by these MP remained unchanged suggesting that these MP carry higher procoagulant potential than MP produced in physiological situation. Heterogeneity in microparticles aminophospholipid content could account for the differences observed in this intrinsic procoagulant activity.

Circulating microparticles seems to be a promising approach in exploration of pregnancy. They constitute a new cellular marker of proinflammatory and procoagulant state in normal pregnancy. In preeclampsia, microparticles with procoagulant potential may participate in the development of microthrombosis in the placental circulation and in the dissemination of procoagulant and proinflammatory informations to vascular cells, thereby exacerbating the generalised inflammatory response. This observation opens new perspectives in understanding the physiopathology of PE and IUGR.

Acknowledgement

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References