Endothelial microparticles: a potential contribution to the thrombotic complications of the antiphospholipid syndrome

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Summary
The antiphospholipid syndrome (APS) refers to persistent antiphospholipid antibodies (aPL) associated with thrombotic and/or obstetrical complications. The endothelial cell is a target of aPL which can induce a procoagulant and proinflammatory endothelial phenotype, as reported both in vivo and in vitro. Microparticle production is a hallmark of cell activation. In the present study, the presence of endothelial microparticles (EMP) in the plasma of APS patients was investigated. To determine if there is a correlation with certain biological and clinical features, EMP levels were measured in thrombosis-free patients with systemic lupus erythematosus (SLE) patients, with and without aPL, in patients with non aPL-related thrombosis, as well as in healthy controls. Compared to healthy subjects, elevated plasma levels of EMP were found in patients with APS and in SLE patients with aPL, but not in SLE patients without aPL or in non aPL-related thrombosis. EMP levels were also associated with Lupus Anticoagulant (LA) detected by a positive Dilute Russell’s Viper Venom time (DRVVT). In parallel, we analyzed the capacity of these plasma to induce vesiculation of cultured endothelial cells. We demonstrated an increase of EMP generated in response to plasma from patients with autoimmune diseases. Interestingly, only APS plasma induced the release of EMP with procoagulant activity. These ex vivo and in vitro observations indicate that generation of EMP in APS and SLE patients results from an autoimmune process involving aPL. Production of procoagulant microparticles in APS patients may represent a new pathogenic mechanism for the thrombotic complications of this disease.

Keywords
Antiphospholipid syndrome, microparticles, procoagulant activity

Introduction
Antiphospholipid syndrome (APS) is a multisystem disorder defined by the association of arterial and/or venous thrombosis or recurrent fetal loss and the persistent presence of antiphospholipid antibodies (aPL), such as lupus anticoagulant (LA) and/ or anti-cardiolipin antibodies (aCL) (1). The syndrome is defined as an isolated entity (primary APS) or is linked to auto-immune pathologies (mainly SLE). Several studies have shown that the binding of aPL to endothelial cells (EC) induces endothelial activation and/or injury, transforming their antithrombotic surface into a prothrombotic one that could contribute to the acquired hypercoagulable state associated with APS (2, 3). However, the pathogenic mechanisms by which these
Recent data obtained from animal models have shown that mice infused with aPL developed thrombi in femoral veins after experimental injury associated with increased adhesion of leukocytes to the vessels. These observations demonstrated that endothelial activation in vivo contributes to the development of thrombosis, and that part of the pathogenic effects of aPL is mediated by endothelial adhesive molecules (4-8). As further in vivo support of aPL mediated endothelial cell activation, increased levels of endothelial markers, such as VCAM-1 or tissue factor were found in the plasma of APS patients with recurrent thrombosis (9-12). In vitro, binding of aPL to cultured endothelial cells induces an up-regulation of surface adhesion molecules and tissue factor expression (13-16). In addition, a subset of aPL that recognizes annexin V has been shown to trigger apoptosis of endothelial cells (17).

It is now well accepted that in response to activation or apoptosis, remodeling of membrane phospholipid bilayer of all eukaryotic cells is associated with the release of microparticles (MP). These vesicles, resulting from an exocytic budding process, express negatively charged phospholipids and cell surface antigens characteristic of the cells of origin. Surface exposure of phosphatidylserine or tissue factor activity provides a catalytic surface that supports the assembly of clotting enzymes complexes, leading to thrombin generation (18). In addition to this procoagulant potential, MP are able to induce various biological responses by a cascade of adhesion and activation of different cell types (19-20). Numerous studies have focused on MP of platelet origin and have found elevated levels associated with prothrombotic or proinflammatory disorders. We have shown that cultured endothelial cells can also shed procoagulant MP after activation by inflammatory cytokines such as TNF (21). In vivo, EMP have been detected in the plasma of healthy subjects and increased levels have been found in the plasma of patients with diabetes, sepsis, idiopathic thrombocytopenic purpura, multiple sclerosis and acute coronary syndromes (22-27). In a preliminary study we detected elevated levels of EMP in patients with lupus anticoagulant, suggesting that APS could be another clinical entity associated to increased endothelial vesiculation (21).

The present study was designed to investigate EMP in patients with primary or secondary APS. In order to investigate their relationship with certain biological and clinical features of APS, EMP levels were also measured in patients with thrombosis-free SLE (with and without aPL) and in patients with non aPL-related thrombosis. In addition, we investigated the capacity of plasma from these patients to induce the release of EMP from cultured endothelial cells and analyzed the procoagulant properties of these EMP.

Materials and methods

Patients
This prospective study was carried out on 111 patients and 25 healthy donors. All gave informed consent to the study, which was approved by the Ethics Committee of our institution. Patients and controls were between 20 and 65 years of age. The subjects were divided into five groups (Table 1). Group 1 (n=35): APS patients, of whom 23 had primary APS and 14 had SLE-associated APS. Group 2 (n=28): SLE aPL+ patients without thrombosis but positive for aPL. Group 3 (n=23): SLE aPL- patients without thrombosis or aPL. Group 4 (n=25): Patients with thrombosis but without SLE, other autoimmune disease or aPL. Group 5 (n=25): sex and age matched healthy subjects (HS). Patients diagnosed as having APS fulfilled the criteria proposed by Harris (28), defined clinically by the occurrence of venous and/or arterial thrombosis and biologically by the persistent presence of aPL. The patients classified as having SLE fulfilled 4 or more revised American College of Rheumatology criteria (29). Thrombotic events included were superficial or deep vein thrombosis and arterial thrombosis. APS patients with obstetrical complications were excluded from the present study. Conventional aPL considered for APS diagnosis were: lupus anticoagulant (LA) and anti-cardiolipin antibodies of IgG and IgM isotypes (IgG-aCL and IgM-aCL). The persistence of aPL was verified on at least two occasions three months apart.

Sample collection
Patients and healthy subjects underwent a complete evaluation of coagulation. For this purpose, blood was collected on 0.129 M sodium citrate tubes (Vacutainer, Becton Dickinson) and platelet-poor plasma was prepared by centrifugation (twice at 3,000 g for 10 minutes). Whole blood and plasma fractions were stored at −80°C until use. In addition to routine parameters (PT, APTT, Fibrinogen, Thrombin time), coagulation screening included measurement of AT, Protein C, Protein S, and search for Leiden mutations of factor V and prothrombin genes. For immunologic aPL detection, blood was collected into plain glass tubes and allowed to clot at 37°C, then centrifuged at 2,000 g for 10 min and serum fractions were stored at −80°C until use. For MP measurement, 5 ml of blood was collected by venipuncture into 0.129 M tri-sodium citrate. Platelet-free plasma was prepared, within 1 h, by two sequential centrifugations 1,500 g for 15 min, followed by a 2-min decantation at 13,000 g to remove all the residual platelets.

Measurement of aPL
Lupus anticoagulant (LA)
Diagnosis of LA was performed according to the recommendations of the Subcommittee on Lupus Anticoagulants/Anti-phospholipid antibodies of the Scientific and Standardization
Committee of the ISTH (30). Screening tests included APTT (APTT bioMérieux, France), Tissue Thromboplastin Inhibition Test (TTIT) using a 1:500 thromboplastin dilution in 25mM CaCl₂ (Neoplastin CI, Diagnostica Stago, Asnières, France) and Dilute Russell’s Viper Venom Time (DRVVT; LAC Screen®, Instrumentation Laboratory, Paris, France).

When prolonged, the screening tests were performed again on a 1:1 mixture of sample and pooled normal platelet-free plasma. Samples with a ratio (mixture clotting time / normal plasma clotting time) ≥ 1.2 for APTT, TTIT and/or DRVVT were considered positive for the presence of an inhibitory activity. In these cases, the phospholipid-dependence of the inhibitor was confirmed by a phospholipid-neutralization procedure using DRVVT (DRVVT LAC Confirm®, Instrumentation Laboratories, Paris, France). A DRVVT normalized ratio [(DRVVT Screen sample / DRVVT Screen normal plasma) / (DRVVT Confirm sample / DRVVT Confirm normal plasma)] ≥ 1.2 was considered positive, indicating the presence of a LA.

Anticardiolipin antibodies
Sera were assayed for IgG- and IgM-class aCL by using an in-house ELISA previously described (31). Values higher than 17 GPLU and 12 MPLU (97 percentiles) were considered positive.

Table 1: Biological and clinical features of the patients.

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
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</thead>
<tbody>
<tr>
<td><strong>Mean age ± SD</strong></td>
<td>38 ±12</td>
<td>42 ±12</td>
<td>39 ±12</td>
<td>44 ±12</td>
<td>40 ±9</td>
</tr>
<tr>
<td><strong>Sex ratio (W/M)</strong></td>
<td>31/5</td>
<td>23/5</td>
<td>24/0</td>
<td>17/10</td>
<td>21/4</td>
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**Antiphospholipid antibodies (%)**

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<tr>
<th></th>
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<th>Group 4</th>
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<tr>
<td>LA</td>
<td>72</td>
<td>64</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>a-CL</td>
<td>44</td>
<td>53</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>a-CL, IgG</td>
<td>40</td>
<td>43</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>a-CL, IgM</td>
<td>4</td>
<td>21</td>
<td>-</td>
<td>-</td>
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</table>

**Thrombophilic abnormalities* (%)**

<table>
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<tr>
<th></th>
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<th>Group 4</th>
<th>Group 5</th>
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<tr>
<td>Thrombotic events (%)</td>
<td>26</td>
<td>10</td>
<td>17</td>
<td>18</td>
<td>8</td>
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<tr>
<td>Venous thrombosis</td>
<td>85</td>
<td>0</td>
<td>0</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>Arterial thrombosis</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>29</td>
<td>0</td>
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</table>

**Treatment (%)**

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<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamine K antagonists</td>
<td>51</td>
<td>3</td>
<td>-</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Anti-platelet</td>
<td>24</td>
<td>28</td>
<td>-</td>
<td>17</td>
<td>-</td>
</tr>
<tr>
<td>Heparin</td>
<td>14</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Corticoids **</td>
<td>29</td>
<td>53</td>
<td>62</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* : Leiden mutations of factor V and Prothrombin, Anti-Thrombin, protein C and protein S deficiencies
** : corticoids ± other immunosuppressors

**Reagents and monoclonal antibodies (mAb) for MP measurements**

Fluorescein isothiocyanate (FITC)-conjugated mAb to vitronectin receptor (anti-αvβ3 or anti-CDS1, clone AMF7, IgG1) and isotype-matched mAb of irrelevant specificity FITC-IgG1 were from Beckmann Coulter Immunotech (Marseille, France). FITC-conjugated annexin V was also purchased from Beckmann Coulter Immunotech. Calibrated latex beads (LB8, 0.8 µm diameter) used to set the MP gate were from Sigma. Before flow cytometry analysis, samples were diluted either in cell culture PBS without Ca++ and Mg++ (Dublecco’s, Life technologies, Paisley, UK) or in appropriate calcium buffer (Beckmann Coulter Immunotech, Marseille, France) when annexin V was used.

**Numeration of EMP in plasma**

EMP were quantitated in plasma as previously described (21). Thirty µl of platelet-free plasma were incubated, during 30 min at room temperature, with 10 µl of FITC-conjugated CDS1 or the corresponding isotype-matched control. Samples were then diluted in 1.5 ml of PBS and a known amount of fluorescent latex beads (Flowcount, Beckmann Coulter Immunotech) was added as internal standard to samples before flow cytometry analysis.
EMP were analyzed on a Coulter® Epics® XL (Coultronics France, Margency) as previously described (21). Using 0.8 µm latex beads, MP were defined as elements with a size inferior to 1 µm and positively labeled with FITC- conjugated CD51. Results were expressed as the number of EMP per ml of plasma.

Incubation of cultured endothelial cells with patient plasma
Ten representative plasmas of each group were selected for the in vitro studies. Human umbilical endothelial cells (HUVEC) were cultured in 24 well plates in standard medium, as previously described (21). At confluence, cells were washed with PBS and incubated with plasma from healthy subjects or patients. Before incubation with cells, plasma were centrifuged during 2h30 at 14000 g and filtered on 0.1µm filters to remove the MP, then diluted 1/5 in culture medium without heparin or fetal calf serum. For control experiments, cells were incubated either with standard heparin-free medium alone or supplemented with TNF (100 ng/ml). After a 48 hour incubation, supernatants were collected for EMP counting and evaluation of their procoagulant activity.

Numeration and procoagulant activity of EMP released by cultured endothelial cells
For EMP quantitation, 10 µl of each conditioned supernatant were incubated with 1 µl of FITC-labeled annexin V for 20 min at room temperature (RT) and then diluted in 500 µl of appropriate calcium buffer. Samples were then analyzed by flow cytometry using the same protocol as defined for EMP numeration in plasma. Results were expressed as the number of annexin V positive EMP per ml of supernatant.

For procoagulant activity measurement, EMP released in supernatant were pelleted by centrifugation at 14000 g during 2h30. The supernatant was discarded and the EMP resuspended in 100 µl of filtered PBS. The number of EMP in the suspension was determined using FITC-annexin V labeling and adjusted with Owren Koller buffer, in order to have the same amount of EMP in each sample. Procoagulant activity was measured, using a chronometric clotting test as previously described (21). Briefly, 50 µl of normal pooled plasma were incubated at 37°C under stirring conditions with a magnetic bar, with 25 µl of either Owren Koller buffer or 25 µl of EMP suspension. Then 75 µl of 25mM CaCl2 were added and the clotting time was measured. For each sample, results were expressed as a normalized clotting time ratio (clotting time of the EMP supplemented plasma clotting time of the control plasma ×100).

Statistical analysis
Statistical analysis was performed using SPSS Software 8.0. Descriptive statistics were calculated for each parameter (mean, standard deviation). First, levels of EMP were compared between the different groups of patients defined by clinical parameters (respectively APS, SLE aPL+, SLE aPL-, Thr aPL- and healthy subjects). Second, the mean levels of EMP were compared between groups of patients defined by the positivity of biological markers. These tests were performed using Anova, Anova-post-hoc and Mann-Whitney tests. For all tests, p < 0.05 was considered as significant.

Results
Demographic description of healthy subjects and patient populations
As shown in Table 1, all groups were comparable according to age and sex ratio, except for the thrombosis aPL- group which had a higher proportion of men. Conventional antiphospholipid antibodies, used for characterization of APS, were explored in all patients and healthy subjects. In the APS and SLE aPL+ groups, LA was the most represented aPL, 72% and 64% respectively, whereas aCL were present in 44% and 53% respectively. The prevalence of thrombophilic markers (Leiden mutations of factor V and Prothrombin, AT, protein C and protein S deficiencies), was comparable in the APS, SLE (aPL+ and aPL-), and non aPL-related thrombosis groups. The number of patients receiving heparin or corticosteroids in each group is listed (Table 1).

Plasma levels of EMP
Plasma levels of EMP differed significantly between groups (overall p value 0.0061) (Fig. 1A). In the APS patients, EMP levels were significantly higher than in healthy subjects (21.3 ± 19.6 vs 7.5 ± 3.9 x 10^3 EMP/ml, p < 0.01) and in the non aPL-related thrombosis (21.3 ± 19.6 vs 11.2 ± 14.5 x 10^3 EMP/ml, p < 0.05) group. No significant difference was observed between primary and secondary APS. EMP levels were also significantly elevated in SLE patients with aPL (20 ± 18.3 x 10^3 EMP/ml, p < 0.01) compared to healthy subjects. In contrast, SLE patients without aPL and patients with non aPL-related thrombosis had EMP levels which were not significantly different from healthy subjects. To further investigate the influence of aPL, EMP plasma levels were analyzed in patients according to the presence of aPL. Patients with aPL displayed a significant increase in circulating EMP compared with patients without aPLs and with controls (20.7 ± 18.8 vs 7.5 ± 3.9 p<0.01 and 12.4 ± 14 p<0.05 respectively) (Fig. 1B).

EMP levels were analyzed according to the positivity of the biological markers. A significant association was found between EMP levels and the presence of LA detected by positive DRVVT values (20.2 x 10^3 per ml for positive DRVVT versus 12.8 x 10^3 for negative DRVVT, p< 0.05, Mann Whitney test). In contrast, no significant association was detected in relation to the positivity of the other parameters studied (LA detected by TTIT, aCL (IgG, or IgM)) even when they were combined. Neither thrombosis nor the presence of SLE or thrombophilic abnormalities affected EMP levels. Moreover, among APS
patients, EMP levels were similar between those with and without thrombophilia. No relationship was found between EMP numbers and antithrombotic or immunomodulatory treatments.

**Effect of patient plasma on MP release by endothelial cells in culture**

In a second step, we investigated the capacity of patient plasma to induce vesiculation in cultured endothelial cells. As a control, the capacity of HUVEC to vesiculate by TNF stimulation (100 ng/ml) was evaluated. This resulted in a more than 3-fold increase in EMP compared to medium alone (1604 +/- 208 vs 460 +/- 86 EMP x 10^3/ml supernatant, p < 0.005, Fig. 2A).

HUVEC stimulation by plasma from healthy subjects led to a moderate, but not significant, elevation of EMP number in the medium (983 +/- 125 EMP x 10^3/ml supernatant). By contrast, APS plasma induced an almost 4-fold increase in EMP (1721 +/- 223 EMP x 10^3/ml supernatant, p<0.01), comparable to that induced by TNF. A significant increase over control plasma was also noted for aPL+ and aPL- SLE plasma (1590...
Elevation was observed for plasma from patients with non aPL- and secondary APS were comparable. In addition, plasma from patients and in SLE patients with aPL. The findings in primary patients may exacerbate cell activation and subsequent thrombotic complications. Whether EMP generation plays a role in the obstetrical complications of APS remains to be investigated.

Although a comparable increase in the number of EMP was generated by the auto-immune plasmas, only APS plasma induced the release of EMP with significantly more procoagulant activity than control. It is well known that endothelial cells are a target for aPL which can induce procoagulant modifications such as inhibition of the anticoagulant functions of annexin V, protein C and S, as well as activation of tissue factor pathway (17,40,41). Our findings suggest that the generation of procoagulant EMP may also contribute to the mechanism by which thrombosis is induced in APS. The fact that SLE patients with aPL did not induce procoagulant EMP is consistent with the existence of various subsets of aPL, with different pathogenicities. This heterogeneity, recently documented by clonal analysis (42), is a feature of aPL. The results of our in vitro experiments support the hypothesis that a subset of pathogenic aPL could be implicated in the generation of EMP disseminating a prothrombotic potential.

In addition to their procoagulant potential, EMP may induce inflammatory and procoagulant effects in other cells. We recently demonstrated that EMP release by stimulated EC in vitro can activate the TF procoagulant pathway by adhering to monocytes (22). Thus, we can speculate that EMP released in APS patients may exacerbate cell activation and subsequent thrombotic complications. Whether EMP generation plays a role in the obstetrical complications of APS remains to be investigated.

Therefore, in vitro generation of procoagulant EMP could represent an indicator of the pathogenicity of aPL and related cofactors present in plasma from APS patients. EMP represent a novel thrombotic pathway which could be involved in the thromboembolic complications of APS and may provide diagnostic information useful in the clinical management of these patients.

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