Diagnostic role of endothelial microparticles in vasculitis

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Objective. Endothelial cells play a central pathogenetic role in ANCA-associated small-vessel vasculitis (AAV). Circulating endothelial cells (CECs), as a marker of endothelial damage, have been shown to be elevated in vasculitis. More recently, endothelial microparticles (EMPs) were found to be increased in active childhood vasculitis. The role of EMP in adult AAV and the relationship between EMP and CEC is unclear.

Patients and methods. We studied 26 patients with AAV, 12 healthy volunteers and 10 patients with IgA nephropathy as disease control. Platelet-poor plasma was ultracentrifuged. MPs were identified and enumerated with flow cytometry, Annexin V, CD62E and CD105 antibodies. Leucocyte- and platelet-derived MPs were also measured. CEC were isolated and enumerated with CD146-driven immunomagnetic isolation.

Results. EMPs are significantly elevated in patients with active vasculitis (CD62E: mean 248 MP/μl; CD105: 121 ± 135/μl) compared with patients in remission/partial remission (CD62E: 55 ± 30/μl, P = 0.001; CD105: 16 ± 12/μl, P = 0.002) and healthy volunteers (CD62E: 66 ± 33/μl, P = 0.002; CD105: 25 ± 26/μl, P = 0.007). The MP count correlates with disease activity measured by the Birmingham Vasculitis Activity Score (BVAS) (CD62E: r = 0.703; CD105: r = 0.445, P < 0.023).

Conclusion. EMPs are elevated in active adult AAV. EMP levels correlate with disease activity and might serve as a marker of endothelial activation and damage. Differential detection of endothelial, platelet- and leucocyte-derived MPs may provide more insight in to the pathogenesis of AAV.

Key words: Microparticles, Endothelium, Laboratory markers, Circulating endothelial cells, ANCA-associated vasculitis.

Introduction

A complex interaction between humoral and cellular elements of the inflammatory system leads to microvascular damage in ANCA-associated vasculitis (AAV), where endothelial cells play a central pathogenetic role. Novel biomarkers of endothelial damage are important to improve diagnostic accuracy and monitor therapeutic success. Traditional soluble markers, such as thrombomodulin and von Willebrand factor, cannot distinguish between endothelial damage and activation [1]. As confirmed recently, measuring ANCA has been disappointing to monitor disease activity and reliable biomarkers are urgently needed [2]. We have previously established circulating endothelial cells (CECs) as a novel laboratory marker of AAV [3, 4]. CECs correlate with disease activity [3] and help to distinguish limited granulomatous disease from active systemic vasculitis [5]. However, the detection of CEC requires endothelial damage and sloughing of cells, which may render this diagnostic tool inaccurate in early stages of AAV, where biomarkers are eagerly needed.

Microparticles (MPs) have received increased attention as universal markers of activation in eukaryotic cells [6]. These vesicles are the product of exocytic budding and consist of cytoplasmic components and phospholipids. The mechanisms of shedding remain poorly understood although changes in the cytoskeleton and exposure of phosphatidylserine on the outer leaflet of the plasma membrane are involved in the process [7]. MPs carry markers of the parent cell, including those induced by activation, apoptosis, cell lysis or oxidative stress. These properties permit detection of specific subpopulations, such as leucocyte-derived, platelet-derived or endothelial MPs (EMPs).

EMPs are increased in many vascular disorders (e.g. hypertension, diabetes mellitus) and autoimmune diseases, which are characterized by vascular damage and thrombosis (e.g. SLE) [8–10]. More recently, EMPs have been found to be increased in childhood vasculitis [11]. There are no current data on EMP in adult AAV. It seems reasonable that EMPs, in contrast to CECs, reflect activation of endothelial cells, which would allow their diagnostic use in early disease stages or reactivation, where endothelial damage and cell sloughing has not yet occurred. In addition, analysis of MPs derived from other circulating cells may provide more insight into the complex pathophysiology of vasculitis. So far, increased neutrophil- and platelet-derived MPs have been described in vasculitis [12].

The purpose of this study was to evaluate MPs of endothelial origin as a biomarker to diagnose and monitor patients with AAV in comparison with CEC. We were also interested in the profile of MPs of platelet and leukocytic origin in patients with AAV.

Patients and methods

Patients

We studied 26 patients with AAV. Nineteen patients had WG, six had microscopic polyangiitis and one patient had Churg–Strauss syndrome. Patients with vasculitis were classified in accordance with the Chapel Hill Consensus Conference [13]. Disease activity was assessed with the Birmingham Vasculitis Activity Score (BVAS) [14]. Thirteen of 26 patients had active vasculitis (BVAS 6–23, median 13), eight patients were in complete remission and five patients in partial remission (BVAS 0–3, median 0). Patients' characteristics are shown in Table 1.

Blood for MP isolation was obtained at baseline in all patients and controls; follow-up measurements were available in 10 patients at different time-points during the first 6 months of their disease course (eight patients with active vasculitis and two patients in partial remission).

Ten patients with biopsy-proven IgA nephropathy (mean estimated GFR 75 ± 39 ml/min) served as disease control group. Twelve healthy volunteers without a history of hypertension,
cardiovascular and inflammatory diseases were also analysed. Patients with systemic infections were excluded.

This study was conducted in accordance with the Declaration of Helsinki and the local Institutional Review Board approved the study protocol. Informed consent was obtained prior to blood collections.

**Blood sampling and preparation of platelet-poor plasma**

Citrated blood from each patient and healthy donor was drawn in an atraumatic fashion from the periphery at the time of diagnosis and in 10 patients at different time-points during their disease course. The storage time of blood never exceeded 1 h after venepuncture. Blood samples were initially centrifuged twice for 5 min at 5000 g at room temperature to receive platelet-poor plasma. Aliquots of 500 µl were stored at −80°C. After thawing, the aliquot of plasma was quickly ultracentrifuged for 60 min at 17 000 g at 4°C to obtain an MP pellet. This MP pellet was reconstituted in annexin V buffer (BD) at 4°C. In order to exclude an effect of the freeze/thawing-process, MP pellets were obtained from fresh and frozen samples. There was no difference regarding the MP count.

**Preparation of MP as a positive control**

Human umbilical vein endothelial cells (HUVECs) were stimulated with TNF-α (BD) to obtain MPs. Monolayers of HUVECs were first cultured in endothelial medium (Cambrex) up to the third passage and incubated for 24 h with 100 ng/ml recombinant human TNF-α. A volume of 800 µl was centrifuged at 17 000 g for 60 min and the supernatant decanted. The MP pellet was reconstituted in phosphate buffered saline and labelled with annexin V-FITC (BD) at 4°C. In order to exclude an effect of the freeze/thawing-process, MP pellets were obtained from fresh and frozen samples. There was no difference regarding the MP count.

**Differential labelling of MP subpopulations**

MP subpopulations were discriminated according to the expression of membrane-specific antigens. The resuspended MP pellet was aliquoted and labelled with mAbs against platelet, endothelial and leucocytic cells (markers are described in detail subsequently). The samples were then incubated in darkness for 10 min at room temperature. Adding an excess of annexin V buffer (200 µl) terminated this step. Analysis by flow cytometry of the EMP thus generated in vitro was used to analyse MP from patient samples.

**Flow cytometry**

After immunolabelling, MP samples were immediately analysed on a Becton Dickinson FACSCanto with FACSDiva software. Dilutions with 0.8 and 3 µm latex beads (Sphero Rainbow Beads, Spherotech, Inc.) were used to confirm the size of MP. For MP quantification, TruCount Tubes (BD) were utilized. The number of MPs per microlitre plasma was calculated with the formula

\[
N = \frac{\text{MP/number of measured beads}}{\text{beads per tube/volume of plasma}} \times \frac{\text{volume of plasma}}{\text{volume of sample}}
\]

**Quantification of CECs**

CECs were isolated from whole blood with Pan-Mouse M450 Dynabeads (Dynal, Oslo, Norway) coated with anti-CD146 antibody (Biotex, Marseille, France) as described previously [16]. CD146 is almost exclusively expressed by endothelial cells [16] and their progenitors [17] with the exception of activated T lymphocytes and some tumour cell lines and trophoblast [18]. After immunomagnetic isolation, cells were incubated with Ulex Europaeus Lectin-1 (UEA-1, Linaris, Wertheim, Germany) (2 mg/ml) for 1 h in darkness. Samples were washed, suspended in buffer and counted with fluorescence microscopy using a Nageotte chamber. UEA-1-positive cells > 10 µm with more than four beads attached were regarded as CECs. Conglomerates were counted as one cell [15].

**Fig. 1.** FACS analysis. The gate of Annexin V-positive MP is seen in the centre. Gates of 0.8 and 3.0 µm Latex beads are given to indicate the size of MP although actual beads are not shown. MPs were identified by size and positive Annexin V-FITC labelling. For quantification we used TruCountBeads, seen in the right upper corner.
Statistical analysis
MP levels were expressed as the mean of MP count per microlitre plasma. As there is normality of our data, one-way analysis of variance (ANOVA) testing was applied to find differences between the groups. Pair-wise comparisons were carried out as a post hoc comparison after all four groups had been compared using ANOVA. As a post hoc test we used the two-sided Dunnett test.

Correlations between BVAS and MP levels as well as potential correlations between leucocytes/platelets/CECs and leucocytic/platelet/EMP levels were evaluated using the parametric Pearson test. For all tests, a P-value < 0.05 was considered significant. Statistical analysis was performed using SPSS 14.0 software (SPSS Inc., Chicago, IL, USA).

Results
MP counts in different patient groups
Absolute MP counts were significantly higher in patients with AAV (mean 928 MP/μl, range 106–2651) compared with patients in remission/partial remission (mean 153 MP/μl, range 55–250), disease control group (mean 149 MP/μl, range 18–404, in IgA nephropathy) and healthy volunteers (mean 177 MP/μl, range 42–325) (two-sided Dunnett test to compare active vasculitis with vasculitis (mean 121 MP/μl, range 7–124 (P < 0.001)) (Fig. 2). A significant increased level of platelet-derived MP in patients with active AAV compared with each group was also found, for CD42a-positive (P = 0.001) as well as for CD62P-positive MP (P = 0.001). MP counts are summarized in Table 2.

Table 2. MPs per microlitre plasma in AAV, IgA nephropathy and healthy volunteers (mean count ± S.D.)

<table>
<thead>
<tr>
<th>Groups of patients</th>
<th>MP markers</th>
<th>Active vasculitis</th>
<th>Remission/partial remission</th>
<th>IgA nephropathy</th>
<th>Healthy volunteers</th>
<th>P-value*</th>
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<tr>
<td></td>
<td></td>
<td>153 ± 72</td>
<td>149 ± 122</td>
<td>177 ± 86</td>
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<tr>
<td>Endothelial MP</td>
<td>Total amount of MP</td>
<td>928 ± 801</td>
<td></td>
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<td>&lt;0.001</td>
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<td></td>
<td>CD 62E</td>
<td>248 ± 198</td>
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<td></td>
<td>CD 105</td>
<td>121 ± 135</td>
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<tr>
<td>Platelet MP</td>
<td>CD 42a</td>
<td>749 ± 761</td>
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<td>0.001</td>
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<td></td>
<td>CD 62P</td>
<td>175 ± 148</td>
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<td>Leucocytic MP</td>
<td>CD 45</td>
<td>280 ± 229</td>
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<td></td>
<td>CD 11b</td>
<td>217 ± 171</td>
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<td></td>
<td>von Willebrand factor</td>
<td>415 ± 371</td>
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<td></td>
<td>CEC</td>
<td>115 ± 103</td>
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<td>0.034</td>
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*Significance between patients with active vasculitis and in remission/partial remission (P < 0.01 for all comparisons between active vasculitis and healthy volunteers).

In patients with AAV, CECs were significantly elevated (mean 115 cells/ml, range 24–400) compared with healthy volunteers (mean 13 cells/ml, range 4–36; P < 0.001). In patients in remission/partial remission, CECs were slightly elevated (mean 43 cells/ml, range 8–164) compared with healthy volunteers, albeit no statistical significance was found (Fig. 3). In patients with IgA nephropathy, CECs did not differ significantly (mean 33 cells/ml, range 4–80) (Table 2) compared with patients in remission/partial remission.

Longitudinal analysis of MPs and CECs
We evaluated serial samples of 10 patients with AAV (eight patients with active vasculitis, two patients in partial remission). Blood samples were drawn at several time points during the course of their disease. Comparing the first and second samples from patients with active vasculitis, mean CD62E-positive EMP declined from 248 to 69 MP/μl plasma and remained stable up to 6 months of immunosuppressive therapy (68 MP/μl). Similar results were found for CD105-positive EMP: 121 MP/μl declined to 25 MP/μl and remained at 23 MP/μl plasma after 6 months of immunosuppressive therapy (Fig. 4).

Regarding CECs, we measured a significant decrease in the number of CECs. In patients with active vasculitis, the initial mean number of CEC was 115 cells/ml, following treatment for 1 month it decreased to 77 cells/ml, declining further to 38 cells/ml blood over the course of 6 months.

Correlation of MP with the BVAS
There was a significant correlation between the BVAS and the total MP (r = 0.7622, P = 0.001), CD62E-positive EMP (r = 0.703, P < 0.001) and CD105-positive EMP (r = 0.445, P = 0.023) (Fig. 5). The amount of von Willebrand factor-positive MP also correlated significantly with the BVAS (r = 0.492, P = 0.007).

A linear correlation between CEC and the BVAS (r = 0.584, P = 0.002) could be observed. Additionally, CRP (r = 0.591, P = 0.001) and white blood cell counts (r = 0.642, P < 0.001) correlated with the BVAS.

Discussion
Novel surrogate markers of endothelial damage in AAV are eagerly awaited because such markers may help to facilitate

FIG. 2. Box plot of CD62E-positive MP levels in patients with active vasculitis (n = 13), in patients in remission/partial remission (n = 13), in patients with IgA nephropathy (n = 10) and in plasma of healthy controls (n = 12). Similar results were found for CD 105-positive MP. ANOVA/two-sided Dunnett test was applied. *Comparison between patients with active AAV and patients in remission; **comparison between patients with active AAV and healthy volunteers.
diagnosis, monitor disease activity and evaluate the effects of treatment. Soluble endothelial markers [19] have seen limited use in vasculitis, not least because some of them depend on renal function [20]. MPs provide another access to endothelial activation and damage in a variety of vascular disorders. Though a consensus protocol to measure MP does not yet exist, flow cytometry-based detection of MP has been shown to be a reliable, reproducible new method [7].

In this study, we used two surface markers to characterize EMPs: CD105 (Endoglin) and CD62 (E-Selectin). CD105 is expressed by endothelial cells, some activated monocytes and leukaemia cells [21]. However, we could not detect CD105-positive MPs released by stimulated mononuclear cells (data not shown). Therefore, CD105 was used primarily as a marker for EMP. CD62E has been shown to be highly expressed by activated endothelial cells and to mediate rolling interactions of neutrophils by ANCA causes adherence to cultured endothelial cells and transmigration across the endothelial layer [23]. Other in vitro data support the involvement of CD62E in the initial steps of vascular inflammation: immunostaining of CD62E in lesions of PAN showed expression in luminal endothelium of early lesions as well as in endothelium of vessels where inflammation was not yet present, but not in advanced lesions or in control specimens [24]. Other potential markers for endothelial activation and damage, such as, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), were analysed by Brogan and coworkers [11] and failed to discriminate between patients with vasculitis and control groups. The usefulness of CD62E and/or CD105 to detect EMP has also been established by other investigators [11, 25, 26].

With the help of these surface markers, we were able to demonstrate that EMPs are significantly increased in adults with active vasculitis compared with patients in remission/partial remission and healthy volunteers/disease controls. Furthermore, EMPs correlate with disease activity measured by BVAS (CD62E: $r = 0.703$; CD105: $r = 0.445$). These findings are consistent with data from a paediatric population with active vasculitis [11] as ratios of MP levels in patients with active vasculitis and patients in remission in our study were in line with these observations in children. Nevertheless, we analysed a relatively small number of patients and a larger study cohort will be needed to confirm our results. Interestingly, active disease resulted in particularly high levels of CD62E-positive MPs, suggesting ongoing release of MPs by activated and/or damaged endothelial cells. Potential confounding factors, such as hypertension, arteriosclerosis, ageing process and smoking status, had no apparent effect on the amount of MP as similar levels of MPs were detected in a paediatric cohort of vasculitis [11].

We were also interested in the relationship between MPs and CECs. In the present study, CECs in patients with active vasculitis were significantly elevated when compared with patients in remission and controls. These findings confirm previous data [3]. However, CECs take longer to decline and do not completely return to normal levels. In comparison, EMPs decline quickly to normal levels after initiation of successful treatment. We speculate that endothelial activation may improve soon after the initiation of immunosuppressive treatment with a subsequent rapid decline of EMP. In contrast, CECs may still undergo apoptosis for longer periods of time, resulting in a prolonged elevation of CEC.
numbers during the repair process of the damaged endothelium. EMPs might thus provide a more sensitive tool to measure disease activity. Moreover, MPs might be useful in the early detection of disease activity as we have followed one patient with active AAV, who had significantly increased EMPs before CECs were detectable. After achievement of remission, EMPs stayed normal. However, longitudinal observations of many patients over long periods are needed to answer these questions.

MPs also play an important role in thrombosis and inflammation [8] and may be a significant pathogenetic factor in AAV. A complex interplay between cytokine-induced expression of proteinsase 3 or myeloperoxidase on the surface of neutrophils, ANCA and endothelial cells is currently regarded as the salient event of AAV. Eventually, activation of neutrophils, respiratory burst, de-granulation and interaction between neutrophils and endothelial cell result in vascular damage [27]. MPs are now regarded as important players at the interface of atherosclerosis and inflammation [8]. MPs are generally capable of inducing cytokine release [28, 29]. Interestingly, PR3 has been found to be on neutrophil MP [12, 30]. Finally, elegant studies in flow chambers have demonstrated that MPs enhance leucocyte rolling [31]. These data suggest that MPs may contribute to the pro-inflammatory status of the endothelium.

We used surface markers for leucocytes and were able to correlate leucocyte-derived MPs with BVAS (r = 0.612). However, neutrophil- or leucocyte-derived MPs need to be used with caution as markers for active vasculitis since neutrophil-derived MPs are elevated in infectious diseases [32]. Functional studies of leucocyte-derived MPs may be more useful to further elucidate the pathogenesis of AAV. Interestingly, EMPs are not elevated in children with a systemic infection compared with children with active vasculitis, supporting the role of EMP as a specific marker of active vasculitis and not systemic infection [11].

There are also several in vitro and in vivo findings supporting the role of MPs in thrombosis. Phosphatidylserine and tissue factor are both exposed on MP outer membranes and are central players in coagulation [33]. Of note, tissue factor has also been found on 80% of the CECs [3] and its expression is stimulated by PR3 in vitro [34]. There are also data suggesting that MPs interact with other coagulation factors [35] and very recent data seem to confirm that tissue factor-independent mechanisms are also at play [36].

We, along with other groups, have observed that platelet-derived MPs are among the highest MPs measured in vasculitic patients [11, 12]. Nevertheless, we could not find a correlation between patients with thrombosis at the time of vasculitis and platelet-derived MPs (data not shown), although a high incidence of venous thrombotic events is known among patients with WG [37]. More patients have to be analysed to answer the question of whether MPs play a role in thrombotic events in vasculitis.

In conclusion, EMPs might serve as a marker of endothelial activation and/or damage in active AAV. This study suggests that MPs are a more sensitive marker than CEC. Our results warrant further studies to confirm the utility of this novel marker in a larger cohort. Longer follow-up periods are also needed to assess the clinical value of EMP as a marker for early vascular damage in active vasculitis and relapse. In addition, functional studies of platelet- and leucocyte-derived MPs and EMPs are needed to dissect how MPs are involved in signalling pathways of cellular cross-talk in vasculitis.

### References


