Evaluation of Circulating Endothelial and Platelet Microparticles in Men with Ankylosing Spondylitis

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ABSTRACT. Objective. To evaluate the profiles of endothelial microparticles (EMP) and platelet microparticles (PMP) in men with ankylosing spondylitis (AS) and healthy subjects. We also aimed to determine whether microparticles (MP) correlate with disease activity, function, and spinal mobility indices.

Methods. There were 82 men with AS and 53 healthy subjects. Subjects with a history of chronic diseases including coronary artery disease, hypertension, diabetes mellitus, and dyslipidemia were excluded. MP were stained with monoclonal antibodies against platelets and endothelial cells and quantified using flow cytometry. MP that were positive for both CD42a+/CD31+ and total CD42a+ were identified as EMP; and MP consisting of CD42a+/CD31+ and total CD144+ were considered EMP.

Results. EMP and PMP were similar between the patient and control groups (p > 0.05). Comparison of patients with AS in the active disease state (BASDAI ≥ 4) and in the inactive state showed that EMP and PMP were not different between the groups (p > 0.05). Correlation analysis revealed no correlation with Bath Ankylosing Spondylitis Disease Activity Index, Bath Ankylosing Spondylitis Functional Index, or Bath Ankylosing Spondylitis Metrology Index. C-reactive protein was significantly correlated with PMP and CD42a+/CD31+ EMP (p < 0.05). Comparison of patients with AS treated with anti-tumor necrosis factor (anti-TNF) drugs, subjects treated conventionally, and healthy controls revealed that PMP and CD42a+/CD31+ EMP were significantly downregulated in patients receiving biological agents.

Conclusion. Circulating EMP and PMP, known to be indicators and mediators of vascular injury, were not significantly altered in men with AS who did not have classical cardiovascular risk factors. Significantly downregulated MP in patients receiving biological agents suggested that anti-TNF treatment may have a beneficial effect on vascular function in AS. (First Release Jan 15 2012; J Rheumatol 2012;39:594–9; doi:10.3899/jrheum.111073)

Key Indexing Terms:
ANKYLOSING SPONDYLITIS
ENDOTHELIUM
CELL-DERIVED MICROPARTICLES
ATHEROSCLEROSIS

Microparticles (MP) are structures that are released from activated or apoptotic cells. They originate mainly from platelets, leukocytes, and endothelial cells1. MP are involved in a wide range of physiological and pathological processes including inflammation, coagulation, antigen presentation, and apoptosis2. The associations among disease pathogenesis, inflammation, and MP, investigators have studied MP in different inflammatory rheumatic diseases3,4,5,6,7,8,9,10,11.

MP may also have a regulatory role in vascular function through various mechanisms and thus they are used as biomarkers for endothelial dysfunction and vascular damage12,13,14. It has been reported that there is increased endothelial dysfunction and vascular impairment in patients...
with ankylosing spondylitis (AS)\textsuperscript{15,16,17,18,19,20}. However, to our knowledge, there are no reports on the relationship between MP and spondyloarthropathies including AS.

We evaluated the profiles of endothelial MP (EMP) and platelet MP (PMP) of men with AS. We also investigated whether the MP correlated with disease activity (Bath Ankylosing Spondylitis Disease Activity Index; BASDAI), function (Bath Ankylosing Spondylitis Functional Index; BASFI), and spinal mobility (Bath Ankylosing Spondylitis Metrology Index; BASMI).

**MATERIALS AND METHODS**

**Patient population.** Our cross-sectional case-control study was conducted between April and September 2010. We studied 82 of 123 adult male patients with AS registered in our department database who had no history of any chronic diseases including diabetes mellitus (current use of medications prescribed to treat diabetes or fasting serum glucose levels ≥ 126 mg/dl), hypertension (average systolic blood pressure ≥ 140 mm Hg; average diastolic blood pressure ≥ 90 mm Hg; or receiving treatment for hypertension), and hyperlipidemia (low-density lipoprotein (LDL) cholesterol ≥ 160 ng/dl or using lipid-lowering medication). All patients satisfied the modified New York criteria for the diagnosis of AS\textsuperscript{21}. Fifty-three healthy controls among the relatives of health professionals and blood donors were also recruited. The same exclusion criteria applied to both controls and patients. Written informed consent according to the Declaration of Helsinki was obtained from all patients before enrollment in the study and approval by the local ethical committee was obtained.

**Blood sampling.** Fasting blood samples were drawn for measurements of fasting glucose, LDL, and standard C-reactive protein (CRP). After collection of citrated fresh blood samples, MP were isolated by double centrifugation at 160 x g for 10 min (platelet-rich plasma) and 1000 x g for 8 min (platelet-poor plasma) at room temperature and immediately stored at −80°C for further analysis\textsuperscript{22}.

**Flow cytometry.** We used TruCount tubes (Becton Dickinson) for determining the absolute number of MP. Briefly, 50 µl of platelet-poor plasma was added to tubes. Then recommended amounts of monoclonal antibodies were included, according to the manufacturers’ instructions. Tubes were incubated 30 min at room temperature in darkness. After labeling, pellets were resuspended in 1 ml phosphate buffered saline and acquired within 1 h. For phenotypic characterization of MP the following cell-specific monoclonal antibodies were used: CD42a-fluorescein isothiocyanate (FITC; Becton Dickinson Pharmingen), CD31-phycoerythrin (PE; Becton Dickinson Pharmingen), and CD144-PE (eBioscience).

Analyses of MP were performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Forward scatter and side scatter of light was set in logarithmic scale and the threshold was set at the forward scatter parameter. Logarithmic green and red fluorescence of FITC and PE were measured through 530/30-nm and 585/42-nm band pass filters, respectively. Data from 75,000 events were acquired and analyzed with CellQuest Pro software (Becton Dickinson). MP gating was based on forward scatter and MP were defined as elements with a size < 1.5 µm relative to standard TruCount beads (4.2 µm).

PMP were defined as events containing total CD42a+ and CD42a+/CD31+. MP consisting of CD42a+/CD31+ and total CD144+ were defined as EMP. The absolute numbers of MP were calculated from the appropriate dot-plot values entered into the following formulas (all sample volumes described here = 50 µl; all illustrated in Figure 1B, 1C): (1) number of total CD42a+ PMP = (number of events in R2 + R3/number of beads collected) x (total number of beads per tube/sample volume); (2) number of CD42a+/CD31+ PMP = (number of events in R2/number of beads collected) x (total number of beads per tube/sample volume); (3) number of total CD144+ EMP = (number of events in R4 + R5/number of beads collected) x (total number of beads per tube/sample volume); and (4) number of CD42a−/CD31+ EMP = (number of events in R1/number of beads collected) x (total number of beads per tube/sample volume). The total number of beads per tube is supplied by the manufacturer and varies among lot numbers.

**Other measurements.** Spinal mobility was assessed by the BASMI\textsuperscript{23}. Patients were also evaluated with the BASFI\textsuperscript{24} and BASDAI\textsuperscript{25}.

**Statistical analysis.** The Kolmogorov-Smirnov normality test was used to determine the distribution pattern of the variables. The majority of data showed non-normal distribution and we used non-parametric tests for statistical analysis. Results are presented as median (range) values. The Mann-Whitney U test was used for comparisons between 2 groups of continuous variables and the Kruskal-Wallis test for comparisons among multiple groups. When the Kruskal-Wallis test produced significant results, the Mann-Whitney U test was used for pairwise group comparisons. Fisher’s exact test was performed for comparison of categorical variables. Spearman rho correlation was used to determine relationships between parameters. The statistical analysis was carried out using the Statistical Package for the Social Sciences, version 13.0 (SPSS, Chicago, IL, USA). A 2-tailed p value < 0.05 was considered significant.

**RESULTS**

Our study group comprised 82 men with AS (median age 39 yrs, range 17–63) and 53 healthy controls (median age 35 yrs, range 24–60). Median disease duration was 13 years (range 1–38). Median BASFI, BASDAI, and BASMI scores were 3.23 (range 0–8.3), 2.45 (range 0–8.72), and 2 (range 0–8), respectively. No patient reported a personal or family history of psoriasis or inflammatory bowel disease. Thirty-four patients (41.5%) had a history of peripheral arthritis, but none had active arthritis at the time of the study. Age, body mass index (BMI), waist circumference, smoking status, fasting glucose, and serum lipids were similar between the patient and control groups (p > 0.05; Table 1). CRP levels were significantly higher in patients than in controls (p < 0.05; Table 1).

**Comparison of patients and controls.** PMP were similar between the patient and control groups (p > 0.05), with median totals for CD42a+ of 7371 (range 938–230565) versus 8472 (range 364–22552) events/µl and for CD42a+/CD31+ of 5896 (range 390–21947) versus 6571 (range 158–21824) events/µl, respectively. Similarly, EMP were also not statistically different between the patients with AS and controls (p > 0.05), with median totals of CD4144+ of 1125 (range 341–6912) versus 1391 (489–4039) events/µl and for CD42a−/CD31+ of 959 (range 107–3259) versus 1006 (range 72–3107) events/µl, respectively. The results of the flow cytometry study are given in Table 1.

**Comparison of active and inactive disease states.** Twenty-four (29.6%) AS patients had active disease (BASDAI ≥ 4). The comparison of active and inactive disease states showed no significant differences regarding EMP, with median total for CD4144+ of 1092 (range 341–6912) versus 1148 (range 348–6517) events/µl (p = 0.9) and for CD42a−/CD31+, 817 (range 212–2446) versus 984 (range 107–3259) events/µl (p = 0.9), respectively; and for the PMP, median total for CD42a+ of 9851 (range
Comparison of patients receiving biological treatments and conventionally treated patients and controls showed that PMP (total CD42a+ and CD42a+/CD31+ events) and CD42a–/CD31+ EMP were significantly downregulated in patients receiving biological agents compared to conventionally treated patients and controls (p < 0.05; Table 2). On the other hand, only total CD144+ events were significantly lower in conventionally treated patients than in controls (p < 0.05; Table 2).

Correlation analysis. Correlation analysis found no correlation between MP and BASDAI, BASFI, or BASMI scores. However, CRP was significantly correlated with median total CD42a+ PMP (r = 0.5), CD42a+/CD31+ PMP (r = 0.4), and CD42a–/CD31+ EMP (r = 0.3). Figure 2 illustrates the correlations between CRP, PMP, and EMP.

DISCUSSION

We demonstrated that EMP and PMP were not different between patients with AS and healthy controls. In addition, subgroup analysis of patients with AS showed that patients treated with biological therapy had significantly reduced EMP and PMP.

EMP are small structures released from endothelial cells that behave as bio-messengers linking inflammation, thrombosis, and angiogenesis. Because EMP can alter vascular homeostasis, they may play a role in the initiation/amplification of numerous inflammatory and thrombotic diseases. EMP and their associations with disease-related factors have been studied in inflammatory rheumatic diseases. Patients with active vasculitis, systemic sclerosis (SSc), and systemic lupus erythematosus had significantly higher levels of EMP than healthy controls. On the other hand, circulating EMP levels were not different between patients with early rheumatoid arthritis (RA) and healthy subjects.

PMP have procoagulant and proinflammatory effects. They also play a role in the modulation of endothelial func-
Similarly to EMP, the role of PMP in AS have not been studied before, to our knowledge. However, PMP have been studied in antiphospholipid antibody syndrome, systemic vasculitis, SSc, and RA. In patients with lupus anticoagulants, the number of MP may correlate with the risk of thrombosis. Significant correlations were observed between PMP and markers of disease activity, including the Birmingham Vasculitis Activity Score, modified Rodnan skin thickness score, and the Disease Activity Score of 28 joints.

Except in inflammatory conditions, it has been shown that cardiovascular risk factors such as hypertension and diabetes mellitus are associated with altered numbers of MP. We assessed EMP and PMP, but found no differences between the patients with AS and healthy controls. In our group, we excluded subjects with chronic diseases, such as

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Table 2. Comparison of patients receiving biological agents and conventional treatment and healthy controls. Data are median (range) values.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Platelet Microparticles, events/µl</th>
<th>Endothelial Microparticles, events/µl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total CD42a+</td>
<td>CD42a+/CD31+</td>
</tr>
<tr>
<td>Anti-TNF treatment, n = 28</td>
<td>5862 (988–28,144)*</td>
<td>3796 (390–18,702)*</td>
</tr>
<tr>
<td>Conventional treatment, n = 54</td>
<td>11,262 (938–230,565)</td>
<td>9512 (715–21,947)</td>
</tr>
<tr>
<td>Controls, n = 53</td>
<td>8472 (364–22,552)***</td>
<td>6571 (158–21,824)***</td>
</tr>
</tbody>
</table>

Significant differences between groups indicated as follows: *patients treated with anti-TNF and conventional agents; ** patients treated with conventional agents and controls; and *** patients treated with anti-TNF and controls. TNF: tumor necrosis factor.

Figure 2. Scatterplots show the correlations between C-reactive protein (CRP) and total CD42a+ platelet microparticles (PMP), CD42a+/CD31+ PMP, and CD42a–/CD31+ endothelial microparticles.
hypothesis and diabetes mellitus, to avoid negative effects of these diseases on the results. In addition, the percentage of smokers and the anthropometric measures (BMI and waist circumference), which may have influenced the findings in MP, were similar between the patient and control groups. The unchanged levels of MP in AS may be the consequence of the following situations: (1) AS does not cause direct vascular damage, unlike other diseases such as vasculitis; (2) the inflammatory activity in AS is not high enough to induce the formation of MP; and (3) our study group may have had relatively mild disease activity and longer disease duration (possibly an effect of excluding comorbidities), which may have caused a less intense inflammatory reaction.

We also evaluated the relationship between circulating MP and markers of disease activity, as well as function and mobility. Although we found no correlation between BASDAI, BASFI, or BASMI and MP, CRP was positively correlated with PMP and CD42a+/CD31+/EMP. Although the strength of the correlation was moderate (probably the result of the small sample size and limited statistical power), the relationship between CRP and MP is noteworthy and might suggest a link between inflammation and MP.

We also showed that patients treated with anti-TNF agents had significantly lower levels of EMP and PMP compared with conventionally treated patients and controls. This finding may be a consequence of the suppression of inflammation or a direct effect of the biological agents. Because increased MP may be associated with vascular injury14,27,28,31, we may speculate that anti-TNF therapy has a beneficial effect on vascular function in patients with AS.

In addition to the small sample size that limited the power of the study, we acknowledge the other limitations: (1) we compared AS patients with healthy subjects and our conclusions are based on this comparison. Inclusion of disease controls, such as those with RA, may be more appropriate in assessing the role of MP in inflammatory rheumatic diseases; (2) inclusion of female patients may also provide information regarding these structures in both sexes; (3) inclusion of AS patients with cardiovascular risk factors may be more useful for understanding the significance of MP in patients with or without cardiovascular conditions; and (4) although flow cytometry is the usual method to quantify MP, the small size of these structures (and difficulty differentiating exosomes, apoptotic bodies, etc.) and the lack of standardization in methodology (e.g., the choice of ultracentrifugation or double-centrifugation) complicate measurement and make the interpretation and comparison of different studies of MP difficult32. Other techniques, including dynamic light scattering, atomic force microscopy, and electron microscopy may be required to measure MP in AS.

Despite these limitations, we showed that circulating EMP and PMP, as indicators and mediators of vascular injury, are not significantly altered in men with AS. Further studies are needed to support our results and the precise role and association of these structures with AS.

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