Circulating endothelial cells in Behçet’s disease: is there a relationship with vascular involvement?

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Objective. Circulating endothelial cells (CEC) are identified in conditions with vascular damage such as systemic vasculitis. Our aim was to investigate if EPC, CEC, and/or its subgroups activated CEC (aCEC) or resting CEC (rCEC) related with vascular involvement in Behçet’s disease (BD).

Methods. In total 60 patients were included in this study, divided into 4 groups: 1) Behçet patients with a history of vascular involvement: vascular BD; 2) Behçet patients with mucocutaneous involvement: mucocutaneous BD; 3) patients with history of thrombosis due to other causes: thrombosis; 4) 20 healthy controls were also included: control group. Percentages of CEC, aCEC, rCEC and EPCs in peripheral blood were measured by flow cytometry.

Results. CEC (3.75 (1.80-7.20), 1.80 (0.70-3.53), 3.50 (1.83-7.23), 2.45 (1.28-4.60)) and aCEC (2.40 (1.28-4.28), 1.10 (0.77-2.20), 3.15 (1.48-7.20), 3.20 (1.15-9.80) levels were did not show a statistically significant difference between groups (p:0.077 and p:0.054, respectively). EPC and rCEC levels were higher in vascular BD and thrombosis groups than mucocutaneous BD and control groups (EPC:10.5 (7.20-18.3), 11.6 (7.30-20.9) vs. 7.15 (5.53-8.25), 10.2 (5.93-18.6), rCEC: 5.35 (3.13-7.90), 6.45 (4.60-10.8) vs. 4.95 (3.05-7.55), 3.40 (1.88-4.30), p:0.042 and p:0.007, respectively).

Conclusion. CEC, EPC, aCEC and rCEC may have role in the assessment of vascular involvement in BD. Longitudinal studies would be needed to identify the utility of these cells for the follow up and risk stratification of BD patients with vascular involvement for recurrences or identify BD patients at risk of vascular involvement.

Introduction

Behçet’s disease (BD) is a systemic vasculitis mostly known with recurrent oral and genital ulcerations, uveitis and mucocutaneous lesions. On the other hand vascular involvement (deep vein thrombosis, cerebral sinus thrombosis and pulmonary artery aneurysm, etc.) is an important clinical finding of disease which may cause mortality and morbidity (1). The mechanisms leading to vascular lesions are incompletely understood (2).

The endothelial layer has the major role in maintaining vascular homeostasis. The endothelial damage may trigger an immuno reaction causing vasculitis (2). Microvascular endothelial cell damage is the hallmark of small-vessel vasculitis and BD is known to effect all sizes of vessels (1, 3). Endothelial progenitor cells (EPCs) are mobilised from bone marrow to peripheral circulation in response to situations causing vascular damage (2). EPCs are involved in both physiologic and pathologic vascular processes (4). Low levels of EPC is supposed to be associated with vascular injury (2).

Vascular damage may cause endothelial cells to detach from the site of injury and release into the circulation (5). Circulating endothelial cells (CEC) are defined in conditions which vascular damage is seen in course of diseases such as systemic vasculitis, coronary artery disease and chronic renal failure. The relation between ANCA-associated vasculitis (AAV) and CEC has been presented in a study by Woyvodt et al. (6). CEC was found to be correlated with active disease and thought to be an indicator of vascular damage in ANCA-associated vasculitis, Kawasaki disease and large-vessel vasculitis (6-8). Depending on the previous studies CEC would reflect disease extent and activity in vasculitis (9). For this reason we have investigated CEC as a possible marker of vascular involvement in BD.

Endothelial cells may be resting or active and the difference may be pre-
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Presented with secretion of chemokines and cytokines or express increased adhesion molecules (10). In a previous study, resting and activated CECs were increased in patients with lymphoma and breast cancer compared with healthy controls and decreased after therapy (11). The clinical significance of these subsets has not been determined yet (10).

Our aim in the current study was to investigate if EPC, CEC, or its subgroups aCEC or rCEC has a relationship with vascular involvement in BD. To answer these questions we have analysed CEC levels in patients with BD, compared them between patients with vascular and mucocutaneous involvement. Also we have compared the results of Behçet patients with patients with thrombosis due to other causes and healthy controls.

Materials and methods

Patients

Current study included 20 Behçet patients with a history of vascular involvement (vascular BD group), 20 Behçet patients with mucocutaneous involvement (mucocutaneous BD group), 20 patients with history of thrombosis due to other causes (thrombosis group) and 20 healthy controls (control group). Behçet patients were diagnosed accord-
ing to the International Study Group criteria (12). Patients with established chronic renal failure, coronary artery disease, diabetes mellitus, hypertension, hyperlipidemia, malignancy and smokers were excluded. Diagnosis of thrombosis was based on doppler ultrasound or computed tomography which were evaluated by experienced radiology specialist. Clinical activity was assessed for activity signs and symptoms according to the BD Current Activity Form (13). The study was approved by local ethics committee (approval number: 25.08.2016/80558721/G).

**CEC analysis**

Blood samples of the patients and healthy controls were drawn into tubes containing ethylene-diamine-tetra-acetic acid (EDTA). A panel of monoclonal antibodies, including anti-CD45 to exclude hematopoietic cells, anti-CD31, -CD34, -CD36, -CD105, -CD106, -CD133 and -CD 146 and appropriate analysis gates were used to enumerate resting and activated CECs and endothelial progenitor cells (EPC) (BD Pharmingen).

A hundred microlitre complete blood was added and incubated for 20 minutes at room temperature in the dark. After incubation for 10 minutes with

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**Fig. 2.** Flow cytometry analysis of one of the patients in Group 2.
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Table I. Demographic and laboratory features of the study population.

<table>
<thead>
<tr>
<th>Group</th>
<th>Vascular BD</th>
<th>Mucocutaneous BD</th>
<th>Thrombosis</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>9 (% 45.0)</td>
<td>12 (% 60.0)</td>
<td>11 (% 55.0)</td>
<td>14 (% 70.0)</td>
</tr>
<tr>
<td>Male</td>
<td>11 (% 55.0)</td>
<td>8 (% 40.0)</td>
<td>9 (% 45.0)</td>
<td>6 (% 30.0)</td>
</tr>
<tr>
<td>Mean ± Standard deviation</td>
<td>Median (Q1 – Q3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>43.55 ± 8.31</td>
<td>46.85 ± 9.43</td>
<td>42.50 ± 15.22</td>
<td>41.65 ± 6.54</td>
</tr>
<tr>
<td>Duration of disease (yrs)</td>
<td>14.90 ± 8.69</td>
<td>11.30 ± 8.80</td>
<td>4.25 ± 3.71</td>
<td>-</td>
</tr>
<tr>
<td>Haemoglobin (mg/dl)</td>
<td>13.0 ± 1.30</td>
<td>14.1 ± 1.60</td>
<td>12.0 ± 2.54</td>
<td>13.8 ± 1.35</td>
</tr>
<tr>
<td>Leucocytes (10³/ul)</td>
<td>8.79 ± 2.47</td>
<td>8.42 ± 2.39</td>
<td>8.25 ± 3.10</td>
<td>7.04 ± 1.66</td>
</tr>
<tr>
<td>Platelet (10³/ul)</td>
<td>261 ± 68.1</td>
<td>250 ± 70.1</td>
<td>294 ± 150</td>
<td>260 ± 62.5</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>28.4 ± 25.3</td>
<td>17.8 ± 15.7</td>
<td>32.2 ± 23.7</td>
<td>20.78 ± 12.8</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>3.53 ± 6.35</td>
<td>1.08 ± 0.970</td>
<td>2.96 ± 4.15</td>
<td>2.32 ± 3.22</td>
</tr>
</tbody>
</table>

BD: Behçet’s disease; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.

erythrocyte lysing solution at room temperature, centrifugation at 1,800 rpm for 5 minutes was performed. Supernatant was removed and washed with phosphate buffer saline (PBS) for two times. Pellet was resuspended with PBS and 1000000 cells were counted with BD FACSCantoII flow cytometry device.

CD146 positive and CD 45 negative cells were defined as CEC, CD146, CD 105 or CD 106 positive cells were defined as activated CECs (aCEC), CD146 positive, CD 105 or CD 106 negative cells were defined as resting CECs (rCEC), CD146 and CD 133 positive cells were defined as EPC (Fig. 1, 2).

Percentages of CEC, aCEC, rCEC and EPCs in peripheral blood mononuclear cells were measured by flow cytometry. Flow cytometric analysis of CEC has been described in detail previously (10).

Statistical analysis
Continuous data are given as mean ± standard deviation, median (Q1-Q3). Categorical data are given as percentage (%). Shapiro Wilk’s test was used to investigate the appropriateness of the data to normal distribution. The Mann-Whitney U-test was used for the two groups, and the Kruskal-Wallis H test was used for the cases with a group number of three groups to non-normal distribution. Pearson’s chi-square analysis was used in the analysis of the cross tables. Box plot was used to see the distribution of the data points per study group. IBM SPSS Statistics 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, v. 21.0. Armonk, NY: IBM Corp.) was used in the implementation of the analyses. A p-value of <0.05 was considered as a criterion for statistical significance.

Results
Age, distribution of sex and duration of disease did not show any difference among groups. Laboratory parameters (haemoglobin, leucocyte and platelet counts, erythrocyte sedimentation rate and C-reactive protein levels were also compared between groups. Haemoglobin levels were lower in Group 3 than all other groups. All remaining parameters did not show any difference between groups (Table I).

Of the 20 patients in vascular BD, 7 patients had deep vein thrombosis (DVT), 4 patients had cerebral sinus thrombosis, 3 patients had pulmonary vasculitis, 1 patient had inferior vena cava thrombosis, 1 patient had thrombosis in coronary arteries, 1 patient had both DVT and pulmonary vasculitis, 1 patient had thrombosis in iliac vein and common femoral vein, 1 patient had cerebral sinus thrombosis, inferior and superior vena cava thrombosis and 1 patient had DVT, hepatic vein thrombosis, inferior vena cava thrombosis and pulmonary artery aneurysm. Eight patients were receiving immunosuppressive treatment at the time of the blood sample collection; 2 mycophenolate mofetil and 6 azathiopurin. The remaining 12 patients’ immunosuppressive therapy was stopped at least 6 months prior to the study.

The thrombosis group consisted of 20 patients with thrombosis related to other causes; 6 patients had systemic lupus erythematosus (SLE) and anti-
phospholipid antibody syndrome, 4 patients had polycythemia vera, 2 patients had rheumatoid arthritis, 1 patient had psoriatic arthritis, 1 had SLE, 1 patient had essential thrombocytemia and 5 patients had thrombosis with unknown causes. Eight patients had DVT, 3 had patients pulmonary thromboembolism, 2 patients had DVT and pulmonary thromboembolism, 1 patient had thrombosis in popliteal, femoral and iliac veins, 1 patient had DVT and splenic vein thrombosis, 1 patient had pulmonary thromboembolism and portal vein thrombosis, 1 had haepatic vein thrombosis, 1 had femoral vein thrombosis, 1 patient had splenic vein thrombosis and 1 had temporal vein thrombosis.

CEC levels did not show a statistically significant difference between groups. EPCs, aCECs and rCECs were also compared between groups. EPCs were higher in vascular BD patients and in thrombosis group than mucocutaneous BD patients and control group (p=0.042). Activated CECs levels did not show a difference between groups (p>0.05). Resting CECs were higher in vascular BD and thrombosis groups than mucocutaneous BD patients and control groups. The detailed analysis of CEC, EPC, activated and resting CECs of groups is given in Table II and Figure 3.

### Discussion

The difference in total CEC numbers was not statistically significant in Behçet patients with mucocutaneous or vascular involvement, patients with thrombosis related with other factors and control group. But EPC and rCEC levels were higher in both patient groups with thrombosis.

CEC elevation in the blood of patients is supposed to be a useful marker for vascular dysfunction (14). Elevated CEC levels were demonstrated in active phase of vasculitis such in ANCA-associated vasculitis and Kawasaki disease (6, 7). In the current study CEC levels tended to be higher in BD patients with history of vascular involvement but the difference did not reach a significant level. Lack of significance might have been caused by: 1. none of the patients had acute thrombosis, 2. all patients received immunosupresive therapy which may decline CEC levels, 3. the small number of patients in each groups. According to the results of the previous studies, increased levels of aCECs may be an indicator of active vascular involvement in BD. But in the current study aCEC levels did not show a difference between groups, even though aCEC levels were higher in vascular BD than mucocutaneous BD. None of the patients had recent vascular events

### Table II. Comparision of CEC, EPC, aCEC and rCECs levels between groups (%).

<table>
<thead>
<tr>
<th>Group</th>
<th>Vascular BD</th>
<th>Mucocutaneous BD</th>
<th>Thrombosis</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>mean ± standard deviation</strong></td>
<td></td>
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</tr>
<tr>
<td>CEC</td>
<td>5.09 ± 4.7</td>
<td>2.52 ± 2.55</td>
<td>4.89 ± 3.83</td>
<td>4.09 ± 4.9</td>
</tr>
<tr>
<td>EPC</td>
<td>13.49 ± 9.38</td>
<td>7.62 ± 4.02</td>
<td>15.6 ± 11.21</td>
<td>13.71 ± 10.1</td>
</tr>
<tr>
<td>aCEC</td>
<td>4.39 ± 5.78</td>
<td>2.24 ± 2.14</td>
<td>8.17 ± 13.26</td>
<td>8.78 ± 13.21</td>
</tr>
<tr>
<td>rCEC</td>
<td>6.44 ± 5.4</td>
<td>5.43 ± 3.49</td>
<td>9.03 ± 7.79</td>
<td>3.52 ± 2.34</td>
</tr>
</tbody>
</table>

CEC: circulating endothelial cell; EPC: endothelial progenitor cell; aCEC: activated circulating endothelial cell; rCEC: resting circulating endothelial cell.
Today assessment of disease activity in BD is mainly based on clinical findings and there is no hint to predict which patient group will develop vascular involvement. Even though we could not show an exact relation, based on the literature, measuring CEC, EPC and rCEC levels may provide to anticipate vascular disease in patients with BD. Also, aCEC may be a marker for active vascular inflammation.

Our study has some limitations; one of them is the design of the study as we do not have a follow-up period to show changes in the number of CEC with disease duration. In addition, we did not have a chance to include any patient with acute thrombosis, which is a major limitation of our study. It would be valuable if we could present the results of BD patients with active thrombosis and compare them with patients who had a history of thrombosis. The relative small number of patients in each group is another limitation.

In conclusion, CEC may be used as a screening test. Increased levels of EPC and rCEC may help us to identify a high-risk patient group for vascular involvement. Resting CECs may be a vascular dysfunction marker in patients with BD.

As far as we know, this is the first study analysing all CEC, EPC, aCEC and rCEC in BD. More research is essential to clearly elucidate the biology of CEC, EPC, aCEC and rCEC in BD.

References