Quantification of circulating endothelial cells in peripheral blood of systemic lupus erythematosus patients: a simple and reproducible method of assessing endothelial injury and repair

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Abstract

Background. Quantification of circulating endothelial cells (CECs) in peripheral blood is developing as a novel and reproducible method of assessing endothelial damage/dysfunction. Accordingly, elevated levels of CECs may be a marker of vascular injury in systemic lupus erythematous (SLE). This study was undertaken to assess the blood level of CECs in SLE and to correlate its level with the activity of the disease and to find out the possibility that the presence of increased numbers of CECs can be used as a marker of immune-mediated vessel damage.

Methods. The study included 33 patients with SLE and 20 healthy controls. They were subjected to clinical examination together with laboratory investigations including complete blood count (CBC), erythrocyte sedimentation rate (ESR), urine analysis, renal function test, C3, C4, ANA, anti-ds DNA antibody, antiphospholipid (IgM and IgG) antibodies and quantification of CECs in blood. CECs were calculated using flow cytometry after staining with a mouse anti-human CD45 antibody (pan-leukocyte marker), mouse anti-human CD146 antibody (endothelial cell marker) and 7-amino-actinomycin D (7-AAD) viability marker. CECs were defined as the live cells with 7-AAD negative, CD45 negative and CD146 positive.

Results. The number of CECs was significantly higher in patients with SLE compared with those in healthy control (mean ± SD 38.6 ± 21.2 versus 7.4 ± 3.4). Furthermore, CECs were correlated positively with SLE disease activity index (SLEDAI) score, ESR and anti-ds DNA. CECs from patients with vasculitic skin lesions, renal and central nervous system (CNS) manifestation were significantly higher than patients free from the previous signs.

Conclusions. An increased number of CECs observed in patients with SLE was associated with the active phase of the disease and may represent a marker of widespread endothelial injury.

Keywords: CD146; flow cytometry; systemic lupus erythematous

Introduction

Organ-specific manifestations in systemic lupus erythematous (SLE) are highly influenced by the inherent characteristic of the vasculature [1]. Widespread activation of the endothelium has been suggested by the observation that even in non-lesional, non-sun-exposed skin from patients with active SLE, the endothelial expression of adhesion molecules and nitric oxide synthase is up-regulated. These findings support the notion that, in SLE, the vascular endothelium in general is 'primed' for injury by activated leukocytes [2].

The study of endothelial injury is difficult due to inaccessibility of the endothelium in humans. However, circulating endothelial cells (CECs) may serve as a new marker for micro-vascular injury [3]. CECs are thought to be mature cells that have detached from the intimal monolayer in response to endothelial injury [4]. Several possibilities can be considered for the mechanism responsible for endothelial detachment. It might be due to apoptosis, mechanical dislodgment of cells, proteolysis of subendothelial matrix proteins or a consequence of complement-dependent injury [5,6].

An increase in CEC number has been recently detected in different diseases, most of which are characterized by prominent vascular pathology such as acute coronary syndromes [7], sickle cell anemia [8], antineutrophilic cytoplasmic antibodies (ANCA)-associated small-vessel vasculitis [9], chronic periaortitis [10], systemic sclerosis [11] and SLE [12].
The original methods for detection of CECs proposed by George et al. [13] and other research groups [3–12] used only CD146 for identifying CECs. However, it is now known that this antigen is expressed on some activated T-cells [14]. In a recent study, Elshal et al. [15] reported, for the first time, that a small percentage of lymphocytes freshly isolated from peripheral blood of healthy individuals express cell surface CD146. Nonetheless, many studies continue to be conducted using CD146 as a sole marker for CECs [3–13]. To this end, the current study was undertaken to accurately enumerate CECs in the circulation of patients with SLE and study their relationship with the development of vascular dysfunction in those patients. Furthermore, we aimed to evaluate the CECs as a monitor for disease activity and thus whether they can be used as a marker of the immune-mediated vessel damage.

**Subjects and methods**

Thirty-three patients who fulfilled the American Rheumatism Association (ARA) criteria for diagnosis of SLE [16] were included in this study. They were recruited from the outpatient clinic of Dermatology and Rheumatology and Rehabilitation departments of Mansoura University. They were 32 females and 1 male, with their mean age 32 ± 10.7 years, range (16–60 years). The duration of the disease ranged from 1 month to 18 years (mean = 5.2 ± 5.1 years). Pregnant females, smokers, patients with diabetes mellitus and patients with cardiovascular dysfunctions were excluded from our study. Of the studied SLE patients, 25 were receiving systemic steroid with a mean dosage of 16.2 ± 11.3 mg prednisolone, and the other 8 patients were either newly diagnosed cases or old cases who stopped the medication for more than 2 months. Twenty age- and sex-matched healthy subjects served as the control group. They met the same exclusion criteria as those of the SLE patients.

All patients underwent complete clinical examination, ECG, echocardiography, lung function tests, chest X-ray, slit lamp examination and fundoscopy. Complete blood count (CBC), erythrocyte sedimentation rate (ESR), urine analysis including 24-h protein in urine, renal function test, C3, C4, ANA, anti-ds DNA antibodies were done using standard laboratory techniques. SLE disease activity index (SLEDAI), a commonly used and validated index of activity status that combines clinical and serological markers of disease severity, was performed [17]. Its mean was 18.75 ± 12.4 (range 2–37) on a scale of 0–105. Antiphospholipid (APL) antibodies (IgM and IgG) were determined in all patients and controls by enzyme-linked immunosorbent assay (ELISA).

### Detection of CECs by flow cytometry analysis [15]

Peripheral blood mononuclear (PBMN) cells were separated from the blood of the patients and healthy control using Histopaque-1077. PBMN cells were then incubated for 5 min with 10 μl of normal mouse serum (Sigma, St Louis, MO, USA) and then resuspended in phosphate buffer saline containing 20 μl of the appropriate antibody. The cells were double stained with a mouse anti-human fluorescein isothiocyanate (FITC)-conjugated CD45 antibody (mouse IgG1K, Becton Dickinson) and mouse anti-human phycoerythrin-conjugated CD146 antibody (mouse IgG1, clone P1H12, Becton Dickinson) to identify CD45− and CD146+ cells, respectively. The isotype control was used to determine nonspecific binding of the lymphocyte subset-specific antibodies and to set the cut-off between fluorescence-negative and fluorescence-positive staining. Ten microlitres of 7-amino-actinomycin D (7-AAD) (Becton Dickinson, USA) was added 15 min before analysis to exclude dead cells.

Stained samples were analysed according to our immunophenotypic criteria using a Becton Dickinson FACSCalibur flow cytometer and Cell Quest software. For data analysis, a size (FSC) versus internal complexity (SSC) dot plot was generated, and a region was drawn around the small, live cell population containing the lymphocyte. Because this region may include variable amounts of thrombocytes and red blood cells, the cell population data obtained from the quadrant statistics (two-colour staining) were standardized for the number of CECs using the sum of CD146+, CD146− and 7-AAD− (live) cells within this region (i.e. CD146+ and CD45−, 7-AAD+ cells were not accounted).

### Statistical analysis

Data were analysed using SPSS (statistical package for social science) version 11. The number and percent as well as mean and standard deviation did descriptive statistics. Unpaired Student’s t-test was used for comparison between groups. The correlation between variables was calculated using Spearman’s correlation coefficient. \( P \leq 0.05 \) was considered statistically significant.

### Results

Clinical and laboratory data of the SLE patients were shown in Table 1. This study included 32 females and 1 male.

<p>| Table 1. Clinical and laboratory data of studied SLE patients |
|-----------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>10</td>
</tr>
<tr>
<td>Skin rash</td>
<td>22</td>
</tr>
<tr>
<td>Vasculitic skin lesion</td>
<td>9</td>
</tr>
<tr>
<td>Arthritis</td>
<td>18</td>
</tr>
<tr>
<td>Arthralgia</td>
<td>14</td>
</tr>
<tr>
<td>Renal affection</td>
<td>14</td>
</tr>
<tr>
<td>Pleurisy</td>
<td>1</td>
</tr>
<tr>
<td>CNS manifestations</td>
<td>11</td>
</tr>
<tr>
<td>Abortion (first trimester)</td>
<td>10/22</td>
</tr>
<tr>
<td>Treatment of systemic steroid</td>
<td>25</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>14</td>
</tr>
<tr>
<td>Leucopenia</td>
<td>22</td>
</tr>
<tr>
<td>Low complement</td>
<td>14</td>
</tr>
<tr>
<td>Anti DNA+</td>
<td>22</td>
</tr>
<tr>
<td>ANA+</td>
<td>26</td>
</tr>
<tr>
<td>Antiphospholipid</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>8</td>
</tr>
<tr>
<td>IgM</td>
<td>6</td>
</tr>
</tbody>
</table>

CNS, central nervous system.
Twenty-two patients (66.6%) had skin rash in the form of malar rash, discoid lesions, recent alopecia or oral ulcers, and nine patients (29.03%) had vasculitic skin lesions in the form of purpura and telangiectasia. Eighteen patients (54.5%) had arthritis in the small joints of the hands, wrists and knees, while 12 patients (36.4%) had polyarthritis. Renal affection in the form of proteinuria ≥0.5 g, haematuria and/or urinary casts was found in 14 patients (42.4%). Central nervous system (CNS) manifestations in the form of seizures, psychosis or severe persistent headache were found in 11 patients (33.3%). Ten out of twenty-two females with previous history of pregnancy (45.5%) reported abortion in the first trimester, and only two of them had recurrent abortion and positive APL antibodies. Twenty-five patients (75.8%) were treated with systemic corticosteroid, while eight patients did not receive any treatment in the last 2 months.

CECs meeting our immunophenotypic criteria are shown in Figure 1, and the mean number of CECs in patients with SLE was significantly higher than healthy control (38.6 ± 21.2, 7.4 ± 3.4, respectively, $P = <0.001$) (Figure 2). The range of CEC number in SLE patients was 10–100 cells/ml, while in the control group it was 3–17 cells/ml.

The number of CECs in patients with SLE was significantly higher in those who had previous history of early abortion (in the first trimester), those having vasculitic skin lesions (purpura and telangiectasia), patients with renal affection and those with CNS manifestations in comparison to patients who were free from previous findings, $P = <0.001$, 0.002, 0.001 and 0.007, respectively. Furthermore, patients with low plasma complement had a significantly higher number of CECs than patients with a normal complement level, $P = 0.011$. Moreover, patients on steroid therapy had a significantly lower number of CECs than those who did not receive steroid treatment, $P = <0.001$ (Table 2). No significant difference in the number of CECs in SLE patients regarding skin rash, joint affection, platelet, WBCs count and APL antibody (IgM or IgG) levels was found (Table 2).
any technique, including flow cytometry (FCM) despite its increasing potential for multicolour detection.

In the present study, CEC numbers in SLE patients were significantly higher than those in healthy control. This finding is consistent with those of the previous studies indicating a higher number of CECs in patients with SLE than in normal control [5,12]. Moreover, in this study CECs were positively correlated with the SLEDAI score and other parameters such as ESR, anti-ds DNA antibody and low plasma complement. There was also tendency towards a higher level in patients with thrombocytopenia and leucopenia but without statistical significance. All these results indicate that CEC levels were higher during the acute phase of the disease and can predict the activity of the disease. This finding was not surprising because in other diseases with vascular injury such as sickle cell disease and ANCA-associated vasculitis, the patients in the steady state had significantly fewer CECs than those in acute crisis, although their CEC levels were still elevated when compared to those of normal subjects [8,18,19].

The detection of high CECs in our patients, particularly those with a higher SLEDAI score, provides compelling evidence for widespread endothelial injury that matches the previous studies done by Clancy et al. [12] and Sesin et al. [1].

Previously, it was shown that increased levels of complement split products are associated with SLE disease activity. In our study, the elevated levels of CECs in peripheral blood correlated positively with low plasma complement (C3 and C4). This finding was consistent with a previous study [12]. Taken together, these results indicate that injury of the vascular endothelium due to complement activation and immune complexes may contribute to the vasculopathy in SLE.

In SLE patients, many clinical signs are indicative of thrombosis such as splinter haemorrhage, livedo reticularis, thrombophlebitis, ulceration, telangiectasia and purpura [20]. Skin vasculitis in the form of purpura and telangiectasia was present in 29.03% of our patients and was positively related to the large number of CECs in our study. 

Sesin et al. [1] showed that the vascular dysfunction characteristic of SLE is associated with dramatically altered distribution of the endothelial protein C receptor (EPCR) with increased soluble EPCR (sEPCR) levels and absent membrane-bound EPCR on the CECs, which may predict and/or reflect vasculopathy and renal injury in SLE [1]. In addition to sEPCR that has pro-inflammatory and pro-coagulant consequences, activated endothelial cells also induce the release of endothelial cell-derived microparticles (EMPs). EMPs are pro-coagulant vesicles formed by the EC membrane after injury or activation [21]. It has been proposed that the generation of EMP in antiphospholipid syndrome (APS) and SLE patients results from an autoimmune process involving APL [22].

Moreover, Clancy et al. [12] found a tendency towards a correlation between CECs levels and anticardiolipin IgG levels in patients with active SLE. On the other hand, Rajagopalan et al. [23] proposed that the APL antibody did not predict endothelial dysfunction and any correlation between the high level of these antibodies and CECs. Furthermore, Meroni et al. [24] suggested that APL antibodies...
per se are not able to induce significant endothelial perturbation in vivo. This may explain why patients consistently positive for APL do display thrombotic events only occasionally. We also found no relation between CECs level and APL antibodies although both are associated with increased risk of thrombotic events. These findings suggest that different molecular mechanisms contribute to the thrombotic complications of this disease.

In the present study, there was a highly significant elevated number of CECs in patients who had previous abortion. Abortion in the first trimester was found in 45% of the females with previous history of pregnancy; however, only two of them had history of recurrent abortion with positive APL antibodies. This result cannot be conclusive, and further studies on large series of lupus patients with history of abortion are needed to prove or disprove this relation.

The effect of treatment with systemic corticosteroid on endothelial function was considered complex, and previous studies yielded conflicting data [25–27]. In a recent study of patients with ANCA-associated vasculitis, a decline of CEC number with successful immunosuppressive treatment had been proved [19]. Furthermore, in our study, patients on steroids had a significantly lower CEC number than nontreated patients.

From this study we concluded that CECs are associated with the active phase of SLE and can be used as a marker of immune-mediated vessel damage. In addition, the increased number of CECs may identify patients at increased risk of lupus nephritis or CNS affection. Furthermore, the observation of low CECs in corticosteroid-treated patients may provide an additional explanation for their efficacy in the treatment of acute flares of SLE. However, further studies are needed to determine the role of CECs in the development of atherothrombosis and cardiovascular disease that is known to have increased number of CECs in patients who had previous abortion.

Conflict of interest statement. None declared.

References

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