Low circulating level of CD133+KDR+cells in patients with systemic sclerosis

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ABSTRACT

Background. Results of previous studies on the level of circulating endothelial progenitor cells (EPCs), which are involved in vascular repair, in scleroderma (SSc) patients have been controversial.

Methods. Enumeration of circulating CD34+KDR+ and CD133+ KDR+ EPCs was performed by flow cytometry. Endothelium-dependent vasodilation was evaluated by changes in flow-mediated dilatation (FMD%) in the brachial artery. Serum level of vascular endothelial growth factor (VEGF) was measured by enzyme linked immunosorbent assay.

Results. SSc patients (n=52) were found to have significantly lower CD133+KDR+EPCs (3.0 vs. 7.0/μl, p=0.001) as well as FMD% (4.8% vs. 7.8%, p<0.001) compared with age- and sex-matched controls (n=52). Among patients who had no concomitant cardiovascular risk factors (n=28), CD133+KDR+ EPC level was significantly lower than controls (3.8 vs. 7.3/μl, p=0.001) and correlated modestly with FMD% (r=0.29, p=0.03). Disease duration was the only determining factor identified for circulating CD133+KDR+EPCs (p=0.03) by logistic regression analysis. Levels of serum VEGF (p=0.92) and KDR expression were not different between patients who had early and intermediate/late disease. Circulating CD34+KDR+ EPCs was not different between SSc patients and controls and did not correlate with any clinical or biochemical parameter.

Conclusion. Lower circulating CD133+KDR+ EPC subset was found in SSc patients and correlated with impaired endothelium-dependent vasodilation in patients without cardiovascular risk factors suggesting a potential role of deficient EPC recruitment contributing to endothelial dysfunction in this disease.

Introduction

Systemic sclerosis (SSc) is a connective tissue disease that is characterised by Raynaud’s phenomenon (RP), sclerodermatous skin changes and internal organ fibrosis. Microvascular abnormalities are common in SSc (1) and are believed to be the sequelae of vascular injury secondary to inflammatory immune processes, ischaemia-reperfusion injury and imbalance between coagulation and fibrinolysis (2). Markers of endothelial activation and damage such as soluble vascular cell adhesion molecule-1 (sVCAM-1) have been found to be elevated in serum of SSc patients (3, 4). Endothelial dysfunction reflected by impaired endothelium-dependent flow mediated dilation (FMD) has previously been demonstrated in SSc patients (5, 6).

Endothelial progenitor cells (EPCs) are bone marrow-derived endothelial precursor cells that contribute to vascular repair (7). This process of rapid endothelialisation of denuded vessels and collateral vessel formation is mediated by vascular endothelial growth factor (VEGF), a potent angiogenic factor that binds to its receptors VEGFR-1 and VEGFR-2 (alternatively known as kinase insert domain receptor (KDR)) on EPCs leading to their mobilisation from bone marrow and subsequent differentiation into mature endothelial cells (8, 9). Deficient recruitment and defective function of EPCs have been reported in cardiovascular conditions including myocardial infarction and associated risk factors (10, 11). However, the role of EPCs in the pathogenesis of
SSc has not been well characterised. There are controversies with regard to increased or decreased circulating EPC levels in SSc patients compared to controls (4, 12-15). In this study, we sought to enumerate circulating EPC subsets and to examine their relationship with endothelial dysfunction evaluated by FMD of the brachial artery, biochemical markers of vascular injury and vascular outcome in our cohort of SSc patients.

**Methods**

**Patients**
The study was approved by the Institutional Review Board of The University of Hong Kong/ Hong Kong West Cluster of the Hospital Authority. A written informed consent was obtained from all participating subjects according to the Declaration of Helsinki. Consecutive SSc patients were recruited from a University affiliated rheumatology clinic. Age- and sex- matched healthy controls were recruited from staff clinic. Subjects who had symptomatic cardiovascular or cerebrovascular diseases were excluded from study. SSc was designated as limited (ISSc) or diffuse (dSSc) depending on the extent of skin involvement according to LeRoy et al. (16). Disease duration was measured from onset of first non-RP manifestations. Early disease was referred to as <3 years and <5 years of disease duration for dSSc and ISSc respectively (13, 17). Baseline demographic data, serological results and medications were documented. Patients who had a history of severe vasospasm leading to the development of ischaemic digital ulcers and/or requiring the use of intravenous iloprost were regarded as having severe RP. Total disease activity score was graded according to the European Scleroderma Activity criteria which includes the total skin score, sclerodema, digital necrosis, arthritis, reduced total lung carbon monoxide transfer factor (TLco), ESR>30 mm/1 h, hypocomplementaemia and deterioration in skin, vascular and muscular/articular condition in the month preceding the assessment (18, 19).

According to these criteria, disease was considered active if the sum of the scores for these items was ≥3.

Echocardiography and lung function test, blood sampling and ultrasound scan of the brachial artery were carried out on the same day. Fasting venous blood sample was obtained to determine serum glucose and lipid levels, erythrocyte sedimentation rate (ESR), c-reactive protein (CRP), sVCAM-1 and VEGF levels and circulating EPC counts. Lung function parameters measured included total forced vital capacity (FVC), forced expiratory volume in 1 second (FEV₁) and TLco. Less than 80% prediction of any of these parameters was considered abnormal. Cardiac involvement was defined as persistently elevated mean pulmonary arterial pressure (PAP) >35 mmHg by echocardiography on at least 2 occasions. Gastrointestinal involvement included clinical dysphagia, reflux oesophagitis requiring the use of proton pump inhibitors or small bowel bacterial overgrowth. Cardiovascular risk factors including current or past history of smoking, hypertension (blood pressure >140/90 or on antihypertensives agents), diabetes mellitus according to WHO definition and hyperlipidaemia (total cholesterol >5.2 mmol/l or on anti-lipid agents) were assessed.

**Measurement of flow-mediated dilation**

Vascular ultrasound was performed with a high-resolution system (Agilent Sonos 5500; Philips, Andover, MA) and a 7.5-MHz linear array transducer by an experienced operator blinded to the studied subjects. All subjects were examined in the fasting state with vascular/articular condition in the month preceding the assessment (18, 19). According to these criteria, disease was considered active if the sum of the scores for these items was ≥3.

Echocardiography and lung function test, blood sampling and ultrasound scan of the brachial artery were carried out on the same day. Fasting venous blood sample was obtained to determine serum glucose and lipid levels, erythrocyte sedimentation rate (ESR), c-reactive protein (CRP), sVCAM-1 and VEGF levels and circulating EPC counts. Lung function parameters measured included total forced vital capacity (FVC), forced expiratory volume in 1 second (FEV₁) and TLco. Less than 80% prediction of any of these parameters was considered abnormal. Cardiac involvement was defined as persistently elevated mean pulmonary arterial pressure (PAP) >35 mmHg by echocardiography on at least 2 occasions. Gastrointestinal involvement included clinical dysphagia, reflux oesophagitis requiring the use of proton pump inhibitors or small bowel bacterial overgrowth. Cardiovascular risk factors including current or past history of smoking, hypertension (blood pressure >140/90 or on antihypertensives agents), diabetes mellitus according to WHO definition and hyperlipidaemia (total cholesterol >5.2 mmol/l or on anti-lipid agents) were assessed.

**Statistical analysis**

Statistical analysis was performed using SPSS 16.0 software. Continuous variables were checked for normal distribution by histogram and probability plot. Normally distributed data was presented as mean±standard deviation (SD). Serum level of EPCs was expressed in median value as the distribution was highly skewed. Categorical data were presented as frequencies and percentages. Comparison between
groups was done by unpaired t test for normally distributed data or non-parametric Mann-Whitney test for non-normally distributed data. Correlations between clinical parameters were evaluated using Spearman’s rank correlation tests. Linear regression analysis was used to evaluate predictive factors for CD133+KDR+ and CD34+KDR+ EPCs as dependent variable. Bonferroni’s correction was applied for multiple comparisons. P-values <0.05 were considered significant in this study.

Results

Clinical characteristics
Fifty-two SSc patients and 52 age- and sex-matched healthy controls (49 female and 3 male) were recruited. There were 41 ISSc and 11 dSSc patients. The mean±SD age of these patients was 51.9±12.5 (range 25-81) years compared to 53.1±10.2 (range 30-78) years for controls (p=0.61). Table I shows the clinical characteristics of these patients. The median disease duration of ISSc patients was similar to dSSc patients (9.0 vs. 7.0 years) (p=0.13). Eleven (21.2%) patients (7 ISSc and 4 dSSc) had early disease. The mean±SD disease activity score of SSc patients was 2.8±1.7 (range 0-6.5). Some patients had total skin score>20 (21.2%, n=11), scleredema (76.9%, n=40), digital necrosis (30.8%, n=16), arthritis (11.5%, n=6), decreased TLCO (25.0%, n=13), ESR >30mm/1st hour (51.9%, n=27), hypocomplementemia (3.8%, n=2) and deterioration in skin (11.5%, n=6), vascular (13.5%, n=7) and muscular/articular (11.5%, n=6) conditions. Some patients were receiving low dose maintenance dose of prednisolone (6.8±3.1mg/day) for the treatment of interstitial lung disease (n=18) and calcium channel blocker for RP (n=35).

Depletion of circulating EPCs in SSc
SSc patients tended to have lower lymphocyte count (1.5±0.7 vs. 1.7±0.6 x10⁹/L, p=0.07) than controls. There were significantly lower median circulating CD133+KDR+ percentage (0.23 vs. 0.40%, p<0.001) and count (3.0 vs. 7.0/μl, p<0.001) in SSc patients compared to normal. There was a trend of lower CD34+KDR+ (11.9 vs. 14.6/μl, p=0.06) EPC count with similar percentages (0.95 vs. 0.89%, p=0.53) in SSc patients compared to controls.

Table I. Demographic characteristics of scleroderma patients (n=52).

<table>
<thead>
<tr>
<th>Demographic characteristics</th>
<th>Mean ± SD (range)</th>
<th>Number (Percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at study (years)</td>
<td>51.9 ± 12.5 (25-81)</td>
<td></td>
</tr>
<tr>
<td>Age at onset (years)</td>
<td>40.6 ± 13.3 (15-71)</td>
<td></td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>12.2 ± 9.3 (1-40)</td>
<td></td>
</tr>
<tr>
<td>Number of patients with early disease</td>
<td>11/52 (21.2%)</td>
<td>ISSc (n=7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dSSc (n=4)</td>
</tr>
<tr>
<td>Female : Male</td>
<td>49:3</td>
<td></td>
</tr>
<tr>
<td>SSc subset (diffuse : limited)</td>
<td>11:41</td>
<td></td>
</tr>
<tr>
<td>Symptomatology / organ involvement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total disease activity score, n&gt;3</td>
<td>2.8 ± 1.7 (0-6.5)</td>
<td>22/52 (42.3%)</td>
</tr>
<tr>
<td>Intersitial lung disease</td>
<td>13/52 (25.0%)</td>
<td></td>
</tr>
<tr>
<td>Elevated Pulmonary arterial pressure</td>
<td>14/52 (26.9%)</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal involvement</td>
<td>21/52 (40.4%)</td>
<td></td>
</tr>
<tr>
<td>Severe Raynaud’s phenomenon</td>
<td>19/52 (36.5%)</td>
<td></td>
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<tr>
<td>Treatment at the time of study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prednisolone</td>
<td>6.8 ± 3.1mg/day*</td>
<td>18/52 (34.6%)</td>
</tr>
<tr>
<td>Penicillamine</td>
<td>19/52 (36.5%)</td>
<td></td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>35/52 (67.3%)</td>
<td></td>
</tr>
<tr>
<td>Angiotensin converting enzyme inhibitor</td>
<td>5/52 (9.6%)</td>
<td></td>
</tr>
<tr>
<td>Statins</td>
<td>1/52 (1.9%)</td>
<td></td>
</tr>
<tr>
<td>Risk factors of cardiovascular disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ever Smoker</td>
<td>6 (11.5%)</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>4 (7.7%)</td>
<td></td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>14 (28.0%)</td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>6 (11.8%)</td>
<td></td>
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</tbody>
</table>

*daily dose among prednisolone users.

Table II. Summary of endothelial dependent vasodilation and circulating EPC level in Scleroderma subsets and patients with different clinical features.

<table>
<thead>
<tr>
<th>SSc subsets</th>
<th>*Number of circulating CD133+KDR+ / CD34+KDR+ EPC (μl)</th>
<th>p-value</th>
<th>*FMD (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All SSc</td>
<td>Patient 3.0 / 11.9, Control 7.0 / 14.6</td>
<td>&lt;0.001</td>
<td>0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ISSc (n=41)</td>
<td>Patient 2.3 / 11.0, Control 7.3 / 13.0</td>
<td>&lt;0.001</td>
<td>0.14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>dSSc (n=11)</td>
<td>Patient 4.6 / 15.0, Control 5.8 / 18.0</td>
<td>0.10 / 0.24</td>
<td>4.1</td>
<td>6.6</td>
</tr>
<tr>
<td>Clinical features</td>
<td>Present 4.6 / 15.0, Absent 3.0 / 11.3</td>
<td>0.58 / 0.54</td>
<td>4.6</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>Present 3.8 / 8.6, Absent 3.0 / 13.5</td>
<td>0.24 / 0.08</td>
<td>4.6</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>Present 3.0 / 15.0, Absent 3.0 / 11.0</td>
<td>0.82 / 0.29</td>
<td>5.2</td>
<td>3.9</td>
</tr>
</tbody>
</table>

*Median value; **Age- and sex- matched to scleroderma patients; dSSc: diffuse scleroderma; EPC: endothelial progenitor cells; FMD: flow mediated dilation; FVC: forced vital capacity; ISSc: limited scleroderma; PAP: pulmonary arterial pressure; SSc: scleroderma
lSSc subset had significantly lower CD133+KDR+ (2.3 vs. 7.3/μl, p<0.001) but not CD34+KDR+ (11.0 vs. 13.0/μl, p=0.14) EPCs than healthy subjects. Circulating CD133+KDR+ (4.6 vs. 5.8/μl, p=0.10) and CD34+KDR+ (15.0 vs. 18.0/μl, p=0.24) EPCs were not different between dSSc patients and controls. There was no difference in CD34+KDR+ (p=0.61) but a trend of lower CD133+KDR+ (p=0.08) EPCs in lSSc patients compared to dSSc patients.

**Impaired FMD% in SSc**
Figure 1 shows the median FMD% of all SSc patients and the SSc subsets. The median FMD% of the brachial artery of SSc patients was significantly lower compared to controls (4.8 vs. 7.6%, p<0.001) suggesting impaired endothelium-dependent vasodilation. The FMD% of lSSc patients was significantly lower (4.9 vs. 8.0%, p<0.001) but only a trend of lower FMD% was observed for dSSc patients (4.1 vs. 6.6%, p=0.06) compared to controls. The FMD% was not different between patients who had early and intermediate/late disease (4.9% vs. 4.6%, p=0.79) and was not related to medication use.

**Elevated sVCAM-1 level in lSSc patients**
lSSc patients were found to have significantly higher serum sVCAM-1 level than dSSc patients (25.4±13.1 vs. 14.8±8.6ng/ml, p=0.01) suggesting a higher state of endothelial activation. This discrepancy was not related to the different disease activity score of these subsets (p=0.81). Serum VEGF levels were not different between SSc subsets and between patients with early and intermediate/late disease.

**Correlations between EPC count, endothelial dysfunction and markers of endothelial injury**
Age was not shown to correlate with CD133+KDR+ (r=0.27) or CD34+KDR+ (p=0.97) EPCs. Circulating CD133+KDR+ (r=−0.28, p=0.04) but not CD34+KDR+ (p=0.59) EPCs in SSc patients were found to correlate modestly and inversely with disease duration (Fig. 2). The levels of expression of KDR on CD34+ (p=0.38) and CD133+ (p=0.27) cells between patients who had early and intermediate/late disease were similar. There were no significant correlations between the EPC subsets, FMD%, VEGF and sVCAM-1 levels. The FMD% of the brachial artery was found to correlate significantly with serum VEGF level in dSSc patients (r²=0.75, p=0.03) (Fig. 1).
3) but not in all SSc patients \( (p=0.77) \) or the ISSc subset \( (p=0.22) \).

**Correlation of EPCs with clinical and vascular outcomes**

Total disease activity score was shown to correlate modestly and inversely with circulating CD133+KDR+ \( (r=-0.29, \ p=0.04) \) but not ESR \( (p=0.91) \) or CRP \( (p=0.63) \) in SSc patients, particularly the ISSc subset \( (r=-0.35, \ p=0.03) \). The EPC subsets were comparable between patients with \( n=14 \) and without elevated PAP and between patients who had digital necrosis secondary to severe RP compared to those without. The EPC subsets were not found to be related to medications but higher CD133+KDR+ \( (4.0 \ \text{vs.} \ 2.3/\mu\text{l}, \ p=0.005) \) EPCs were found in patients on penicillamine who tended to have shorter median disease duration \( (8.5 \ \text{vs.} \ 11.5 \text{years}, \ p=0.09) \), compared to whose not on this medication.

**EPCs in SSc patients without cardiovascular comorbidities**

Twenty-eight SSc patients \( (7 \text{ dSSc and 21 ISSc}) \) and their age- and sex-matched controls \( n=28 \) were further analysed after elimination of those who had concomitant cardiovascular risk factors. These SSc patients were found to have significantly lower CD133+KDR+ EPCs \( (3.8 \ \text{vs.} \ 7.3/\mu\text{l}, \ p=0.001) \) but similar CD34+KDR+ EPCs \( (p=0.43) \) compared to controls. FMD% was shown to correlate modestly with CD133+KDR+ \( (r=-0.29, \ p=0.03) \) but not CD34+KDR+ \( (p=0.84) \) EPCs among these patients. The EPC subsets were not found to correlate with total disease activity score, ESR or CRP. The EPC subsets were not different between patients who had low TL_CO2, digital necrosis secondary to severe RP and elevated PAP compared to those who did not have these manifestations.

**Multiple logistic regression analysis**

Using CD133+KDR+ EPC count as dependent variable, variables identified in univariate analysis with \( p \)-value \(<0.1 \) including penicillamine, total disease activity, low FVC, disease duration and SSc subset were analysed as independent variables in multiple logistic regression analysis. Disease duration was the only factor found to be associated with EPC counts \( (p=0.03) \).

**Discussion**

Our study showed that the CD133+KDR+ EPC subset was significantly lower in SSc patients compared to age- and sex-matched controls. This was further confirmed by analysing only those patients who did not have concomitant cardiovascular risk factors. However, the CD34+KDR+EPC subset was only marginally lower than control subjects. Disease duration was the only significant determining factor for CD133+KDR+EPC count found in multiple logistic regression analysis in an inverse relationship. Indeed, previous studies have demonstrated higher EPC level in early SSc compared to long-standing disease \( (4, 12, 13, 15) \). Reduced level of angiogenic factors \( (24, 25) \), impaired expression of VEGFR-1 by EPCs \( (26) \) have also been reported in SSc patients with long standing disease. However, our patients with early and intermediate/late disease had similar levels of serum VEGF and KDR expression on EPCs suggesting alternative mechanisms. Bone marrow derived mesenchymal stem cells have been reported to be decreased with impaired function in these patients \( (14, 17, 27) \).

We found significant correlation between circulating CD133+KDR+EPCs and FMD% among SSc patients who had no concomitant cardiovascular factors suggesting a potential role of deficient EPC recruitment in endothelial dysfunction. Endothelial dysfunction has been associated with cardiovascular risk factors \( (28) \) as well as SSc disease \( (5, 6, 29, 30) \). Elevated serum pro-inflammatory nitric oxide metabolites in SSc patients have also been suggested to contribute to endothelial dysfunction by oxidative stress \( (31, 32) \). Impaired endothelium-dependent vasodilation was predominantly found in our ISSc patients. This may be related to the small sample size of dSSc subjects or a lower state of endothelial activation reflected by lower serum sVCAM-1 in these patients. Nevertheless, the correlation between impaired endothelium-dependent vasodilation and VEGF level in the dSSc subset suggested endothelial dysfunction may account for the deficient release of angiogenic factors in some patients.
The association between EPCs and disease activity (4, 13, 14) or severity (15) remains controversial. We only found a weak correlation between CD133+KDR+ EPCs with total disease activity. EPC subsets were not shown to be related to vascular outcomes including elevated PAP or digital necrosis secondary to severe RP in our SSc patients. Furthermore, discrepancies have also been reported in EPC levels in SSc patients (12, 13, 15, 27). This may chiefly be accounted by variations in definition of EPCs together with different composition of cohorts comprising of patients with variable disease duration. Indeed, CD133+KDR+ and CD34+KDR+EPCs represent subgroups of putative EPCs in the circulation. More mature EPCs are negative for CD133 but positive for CD34 and KDR (33, 34). The CD34+EPCs population we measured may have included CD34 expressing mature endothelial cells derived by shedding. Thus, recently, the EULAR Scleroderma Trials and Research (EUSTAR) group recommended the use of three colour staining involving CD133, KDR and CD34 by flow cytometry in combination with a viability marker to define EPCs in peripheral blood (35). Since our research work was performed before the issue of the EUSTAR recommendation, further work is required using EPCs as defined by EUSTAR needs to be carried out. Our study has, nevertheless, raised a concern on the practicality of using EPCs as vascular biomarkers in clinical use in SSc because of their susceptibility to influence from concomitant cardiovascular risk factors. In addition, circulating EPC levels have also been found to vary during different phases of menstrual cycle (36). This may add to difficulties in studying EPCs in SSc patients as irregular menstrual cycle has been reported to be common among female patients (37). In conclusion, we found decreased circulating CD133+KDR+EPCs in association with impaired endothelium-dependent vasodilation in SSc patients suggesting defective mechanism for vascular repair in this disease. Our study was limited by the small sample size with multiple comparisons for different outcomes. Studies into the role of EPCs in the pathogenesis of SSc may have treatment implications as direct infusion of EPCs (38) or mobilization of EPCs by statins (39) or granulocyte colony-stimulatory factor (40, 41) may offer potential treatment options for vasculopathy associated with this disease. Future studies with larger patient cohorts are warranted to examine for subset difference in circulating EPC counts, their relation with disease activity and various vascular outcomes in SSc patients.

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