Supporting cells in neovascularization: study on candidates for cellular therapy
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Elevated endothelial progenitor cells during painful crisis in sickle cell disease

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Abstract

Sickle Cell Disease (SCD) is characterized by recurrent micro vascular occlusion and ischemia that may lead to an angiogenic response. Since circulating endothelial progenitor cells (EPC) have been suggested to modulate neovascularization, we studied EPC mobilization in consecutive patients with SCD. During vaso-occlusive events, numbers of circulating EPC were higher than in steady state (42 (0–186) cells/ml vs. 10 (0–28) cells/ml; $P<0.005$). Levels of stromal cell derived factor-1α (SDF-1α), erythropoietin (EPO), soluble vascular cellular adhesion molecule-1 (sVCAM-1) and vascular endothelial growth factor (VEGF) were found increased during crises. Only SDF-1α correlated with EPC numbers, while no correlation was found with manifestations of organ damage.
Introduction

Sickle cell disease (SCD) is characterized by chronic haemolytic anaemia and recurrent (micro)-vascular occlusion leading to tissue ischemia and reperfusion injury\(^1\). Tissue ischemia is at least one of the factors leading to a pro-angiogenic state in SCD\(^2\). This SCD associated pro-angiogenic response is likely involved in tissue repair following ischemic damage by inducing neo-vascularization. However abnormal angiogenesis may be of importance in the formation of aberrant blood vessels, leading to complications such as sickle cell retinopathy and Moyamoya disease\(^3\), while the process of vascular remodelling can also be associated with complications such as pulmonary hypertension and stroke\(^4\). Therefore, a better understanding of the process of angiogenesis in SCD seems of importance for identifying both new parameters for risk assessment as well as potential targets for therapy.

Recently the important role of endothelial progenitor cells (EPC) in angiogenesis has been recognized\(^5\). EPC are defined as hematopoietic stem cells (HSC) with VEGF receptor-2 (VEGFR-2) expression and can be detected in peripheral blood by flow cytometry. Upon mobilization by pro-angiogenic factors such as vascular endothelial growth factor (VEGF), stromal cell derived factor-1α (SDF-1α), erythropoietin (EPO) and granulocyte-colony stimulating factor (G-CSF)\(^6\textsuperscript{-8}\), EPC can take part in neovascularization after ischemic damage. In order to further characterize the angiogenic response in SCD, we determined for the first time the numbers of circulating EPC at steady state and during painful crises. Furthermore, serum levels of VEGF, SDF-1α, PIGF, EPO, IL-8 and sVCAM-1, known to play a role in mobilization and homing of EPC, were determined.

Material and Methods

Patients

Consecutive adult (18 years and older) clinically asymptomatic patients with SCD (HbSS, HbSβ\(^0\)-thalassemia, HbSβ\(^+\)-thalassemia and HbSC confirmed by high performance liquid
chromatography) visiting the outpatient clinic and sickle cell patients admitted for a painful crisis in the Academic Medical Centre (AMC, Amsterdam, The Netherlands) were eligible for the study. A painful event was defined as typical musculo-skeletal/abdominal pain not explained otherwise. Exclusion criteria were: documented active infections, pregnancy or the presence of any acute cardiovascular complication. For data analysis, the more severe genotypes HbSS and HbSβ0-thalassemia (HbSS/HbSβ0-thalassemia) and the relatively milder genotypes HbSC and HbSβ+-thalassemia (HbSC/HbSβ+-thalassemia) were grouped together. Healthy volunteers served as controls. All patients and controls gave written informed consent. Patient histories were obtained by chart review. The protocol was reviewed and approved by the local medical ethical committee and conducted in agreement with the Helsinki declaration of 2000.

Flow Cytometry
Within 24 hours of venous blood collection, samples were incubated in a Trucount tube (two tubes per subject, 100μl peripheral blood/tube, BD Biosciences) with antibodies against CD45 (PerCP-labelled, BD Biosciences), CD34 (APC-labelled, BD Biosciences) and VEGFR-2 (Pe-labelled, R&D Systems, Minneapolis, MN, USA). NH4Cl solution was added to lyse red cells and the samples were immediately measured on a flow cytometer (FACSCANTO, BD Biosciences). Data were analysed using FACSDIVA software. For enumeration of circulating HSC (CD45 dim/CD34 bright) and EPC (HSC with VEGFR2 expression), flow cytometric analysis was performed using a multiparametric gating strategy that avoids inclusion of mature circulating endothelial cells, which are also positive for CD34 and VEGFR-2.

Humoral factors
Serum levels of EPO, SDF-1α, interleukin-8 (IL-8), VEGF, PIGF and sVCAM-1 were measured by enzyme-linked immunosorbent assays according to manufacturers procedures (R&D Diagnostics, Minneapolis, MN, USA).

SCD-related complications
The frequency of painful events in the last five years prior to sample collection was determined by chart review and the presence of SCD related organ damage (pulmonary
hypertension, leg ulcers, microalbuminuria, renal failure and retinopathy) was screened for as previously described.

Data analyses and statistics
For multiple group comparisons of continuous variables the Kruskal–Wallis test was employed. The Mann–Whitney U-test and the Wilcoxon Signed Rank test were used for comparisons between two groups and paired analysis within groups respectively. For correlation studies, the Spearman Rank correlation coefficient (rs) was determined. Statistical software (Statistical Package for the Social Sciences (SPSS), version 14.0, SPSS Inc.) was used. Data are presented as medians with interquartile ranges (IQR), unless stated otherwise, p-value < 0.05 was considered to indicate a statistically significant difference or correlation.

Results
Sixty six consecutive steady state (age 28 (21 - 38), 35 women, 49 HbSS/HbSβ⁰-thalassemia and 17 HbSC/HbSβ⁺-thalassemia) and 36 consecutive painful vaso-occlusive events in 23 patients (age 28 (21 - 37), 13 women, 15 HbSS/HbSβ⁰-thalassemia and 8 HbSC/HbSβ⁺-thalassemia) were included in this study.

Circulating progenitor cells:
Both in steady state and controls, number of EPC was measured in most cases below the detection level of 20 EPC/mL. The numbers of EPC that were measured were comparable between steady state patients and controls. However, during painful crisis EPC numbers increased significantly (table I). A trend to higher numbers of circulating EPC was detected in paired analysis of 12 cases in which EPC levels were measured both in steady state and during painful crisis (26 (0-149) vs. 0 (0-0); P = 0.051). EPC did not differ between HbSS/Hbβ⁰-thalassemia and HbSC/HbSβ⁺-thalassemia patients (data not shown). Numbers of circulating HSC were comparable between healthy donors, steady state patients and patients with painful events.
### Table 1. Endothelial progenitor cells (EPC) and hematopoietic stem cells (HSC)

Data presented as medians (interquartile ranges). *p* < 0.001, EPC numbers during painful event higher than in steady state. † *p* < 0.001 EPC numbers in healthy donors comparable with steady state patients and lower than patients with painful events.

*Paired analysis of EPC and HSC numbers during painful events and steady state in 12 patients.

<table>
<thead>
<tr>
<th></th>
<th>SCD (n = 36)</th>
<th>Healthy donors (n = 66)</th>
<th>P-value paired*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPC/ml</td>
<td>42 (0 – 186)</td>
<td>0 (0 – 0)*</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HSC/μl</td>
<td>2.3 (1.3 – 4.1)</td>
<td>1.8 (1.1 – 3.4)</td>
<td>1.3 (0.8 – 2.5)</td>
</tr>
</tbody>
</table>

**Humoral factors:**

Serum levels of SDF-1α, sVCAM-1 and VEGF were significantly higher during painful crisis as compared to steady state, whereas EPO, IL-8 and PIGF levels were similar. Paired sample analysis in 7 patients from whom both painful crisis and steady state samples were available revealed significant increments in SDF-1α, sVCAM, VEGF and EPO (Table II, *P* < 0.05) during painful crisis. EPC numbers were significantly correlated only with SDF-1α serum levels (*r* = 0.5; *P* = 0.014).

**SCD-related organ damage**

No correlation between circulating EPC numbers and the presence of pulmonary hypertension, leg ulcers, microalbuminuria, retinopathy or frequency of painful events was detected (data not shown).
<table>
<thead>
<tr>
<th></th>
<th>Steady State (n = 54)</th>
<th>Crisis (n = 30)</th>
<th>P-value</th>
<th>P-value Paired (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPO (mIU/ml)</td>
<td>78 (50–156)</td>
<td>93 (50–159)</td>
<td>NS</td>
<td>0.028</td>
</tr>
<tr>
<td>SDF-1 α (pg/l)</td>
<td>2925 (1901–6719)</td>
<td>24345 (4172–27424)</td>
<td>0.0001</td>
<td>0.028</td>
</tr>
<tr>
<td>IL-8 (pg/l)</td>
<td>23 (11–40)</td>
<td>31 (22–47)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>sVCAM-1 (ng/ml)</td>
<td>1044 (857–1308)</td>
<td>1346 (1133–1629)</td>
<td>0.001</td>
<td>0.028</td>
</tr>
<tr>
<td>PIGF (pg/ml)</td>
<td>21 (13–26)</td>
<td>16 (12–23)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>248 (117–615)</td>
<td>486 (394–1042)</td>
<td>0.001</td>
<td>0.018</td>
</tr>
</tbody>
</table>

**Table II. Angiogenic growth factor serum levels in SCD patients both in steady state and during painful events**

Data presented as medians (interquartile ranges). *Paired analysis during painful events and steady state in 7 patients.

**Discussion**

Bone marrow derived endothelial progenitor cells have been suggested to play an important role in neovascularization and vascular repair\(^5\). Mobilization of EPC has been described in patients with clinical conditions characterized by acute ischemia or a strong inflammatory response and is mediated by the release of pro-angiogenic growth factors such as EPO and VEGF\(^5\). Patients with SCD are characterized by a pro-angiogenic response in steady state which further increases during painful vaso-occlusive events\(^2\). In agreement, we report here a significant increase in circulating EPC in sickle cell patients during vaso-occlusive events although no increase was observed in patient in steady state conditions. Interestingly, no difference in HSC was observed between patients in steady state and those with painful events, indicating a different mobilizing mechanism for EPC and HSC.

Several growth factors have been suggested to mediate the mobilization of EPC\(^7\)\(^-\)\(^9\). Although EPO is chronically elevated in SCD and has been demonstrated to induce EPC mobilization\(^7\), we found no correlation between the number of circulating EPC and EPO serum levels ruling out an important role of EPO in EPC mobilization in sickle cell patients. This might be explained by the fact that the mobilizing effect of EPO is highly dependent on nitric oxide (NO) synthesis\(^10\) which is known to be strongly reduced in
SCD\textsuperscript{11}. Despite the significant increase in the serum levels of VEGF and sVCAM serum levels during vaso-occlusive crisis only SDF-1α serum levels appeared to correlate with the numbers of circulating EPC during sickle cell crisis. SDF-1α is considered to mediate the homing of circulating progenitor cells and has demonstrated to be predominately expressed at sites of ischemia\textsuperscript{12}. Interestingly, in diabetic mice with reduced NO synthesis, SDF-1α has shown to play a major role in the mobilisation and homing of EPC\textsuperscript{10}.

In contrast to our previous observation, no difference in IL-8 serum levels could be found between steady state and vaso-occlusive crisis. However, given the known short duration of the IL-8 peak levels in sickle cell crisis, these differences might be explained by the fact that in our study the blood samples were taken after admission and during treatment of the vaso-occlusive event while the previous observation was done in blood samples taken immediately at presentation. We can therefore not exclude a role for IL-8 response in EPC mobilisation in the present study.

In conclusion, in sickle cell disease an increased number of circulating endothelial progenitor cells can be found confirming the pro-angiogenic state and neovascularization response upon vaso-occlusive ischemia in these patients which correlated with SDF-1α levels. Future studies should reveal whether the number of circulating EPC can be used as a biomarker for the (prediction of) severity of SCD.
References
