Novel visualization techniques towards identification of atherosclerotic patients at risk

Jager, Nynke

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CHAPTER 2

Endothelial Progenitor Cells and cardiovascular risk in patients with Early Rheumatoid Arthritis

Nynke A. Jager, Lodewijk de Groot, Steven Wenker, Andries J. Smit, Cees G.M. Kallenberg, Marcel D. Posthumus, Marc Bijl, Johanna Westra

Submitted for publication
Abstract

OBJECTIVES The incidence of cardiovascular disease (CVD) is increased in rheumatoid arthritis (RA) patients. Endothelial progenitor cells (EPCs) are supposed to play a role in repair of endothelial damage. The aim of this study is to relate EPC numbers to endothelial activation and dysfunction preceding atherosclerosis in early RA patients and to investigate whether a diminished number of EPCs might be associated with increased cardiovascular risk.

METHODS EPC numbers were measured from twenty-seven RA-patients, with recent (≤ 1.5 year) onset of disease and 15 healthy controls (HC) by flow cytometry using anti-CD34 and anti-CD133 antibodies. Colony-forming units (CFUs) were counted by culturing mononuclear cells on fibronectin coated plates. Serum endothelial activation markers, small artery elasticity (SAE), disease activity (disease activity score of 28 joints (DAS-28)) and traditional risk factors for atherosclerosis were assessed.

RESULTS Numbers of EPCs, CFU counts and SAE were decreased in RA patients compared to HC (p=0.02, p<0.0001, and p=0.02 respectively), while endothelial activation markers von Willebrand factor (vWF), soluble vascular cell adhesion molecule (sVCAM)-1 and vascular endothelial growth factor (VEGF) were increased (p=0.0002, p=0.05 and p=0.04 respectively). Numbers of EPCs and CFUs were correlated to SAE and inversely correlated to DAS-28 score. EPC numbers were inversely correlated to angiopoietin-2 (ANGP-2) and sVCAM-1 levels.

CONCLUSION CFU counts and EPC numbers are reduced in early RA patients compared to HC, are inversely related to RA disease activity, and correlated to endothelial activation markers and SAE. EPC shortage in peripheral blood might play a role in accelerated development of atherosclerosis in RA patients.

Keywords: Early Rheumatoid Arthritis, Endothelial Progenitor Cells, Colony forming units, Serum endothelial activation markers, Disease Activity Score
Introduction

Rheumatoid arthritis (RA) is a chronic disease characterized by inflammation of joints, but it can also affect a variety of other organ systems (1). RA is associated with an increased mortality. The expected survival of RA patients is likely to decrease by 3-10 years comparing to healthy controls and is related to the severity of the disease and the age of disease onset (2).

The leading cause of death in RA patients is cardiovascular disease (CVD). Cardiovascular mortality is increased in RA patients relative to the general population (1,3). The higher prevalence of atherosclerosis in RA patients is thought to be due to overlapping factors in the pathogenesis of RA and atherosclerosis, such as T-cell activation, macrophage - and mast cell activation, and production of proinflammatory cytokines as TNF-α and IL-6. The systemic inflammatory state results in endothelial activation and endothelial dysfunction (4). However, adequate biomarkers to identify patients at risk for CVD are still needed.

Endothelial cell activation is represented by the production of vascular endothelial growth factor (VEGF) and the release of angiopoietin-2 (ANGP-2), which antagonizes binding of angiopoietin -1 (ANGP-1) to the Tie-2 receptor, sensitizing the endothelial cells to up-regulate and release adhesion molecules (5) as soluble vascular cell adhesion molecule-1 (sVCAM-1), thrombomodulin (TM or CD141) and von Willebrand Factor (vWF). Subsequently, endothelial dysfunction will occur which results in increased 'stiffness' of the arterial wall, measured using tonometry of the radial artery by pulse wave analysis (PWA), which is recalculated to Small Arterial Elasticity (SAE) (6). Endothelial dysfunction might be regarded as a first sign of endothelial damage and, as such requires recovery.

Endothelial progenitor cells (EPCs) appear to be important in endothelial repair (7). EPCs can repair or form new blood vessels through two different mechanisms: by endothelial sprouting from a preexisting capillary network (angiogenesis) or by vasculogenesis, which refers to blood vessel formation from EPCs differentiating in situ (8). Therefore, the number and functionality of circulating EPCs may be an important factor contributing to the recovery in the atherogenic process (9). EPCs represent a population of human bone marrow-derived cells expressing CD34, vascular endothelial growth factor receptor-2 (VEGFR-2 or KDR) and CD133, which is a stem cell subset marker (10). There are several techniques to study EPCs. One is detection by cell surface phenotype using fluorescent labeled antibodies and flow cytometry (11). However, this method might fail to discriminate hematopoietic cells from EPCs as some subpopulations of hematopoietic progenitor cells express the same surface markers. For this reason another technique was developed by Hill and co-workers which tests the ability of human peripheral blood mononuclear cells (PBMCs) to develop into colony-forming units (CFUs). PBMCs were plated on fibronectin-coated dishes; next nonadherent cells were replated to quantify the emergence of CFU (called CFU-Hill) seven days later (12). This in vitro adhesion and growth method exhibit more endothelial characteristics (expression of CD31, Tie-2) and is therefore more specific,
but also more complex and time-consuming than flow cytometry. In the present study both methods were used.

The aim of this study is to evaluate whether the increased endothelial activation and dysfunction of RA patients is related to decrease in EPC numbers. We hypothesize that in RA patients, in comparison to healthy controls, EPC numbers in the peripheral blood are lower due to disease activity. Subsequently, endothelial repair mechanisms are less active and lead to endothelial dysfunction, reflected by a decrease in SAE and an increase in traditional risk factors for atherosclerosis.

Materials and Methods

Study design
RA patients have a higher risk to develop cardiovascular disease over time (13). To prevent confounding effects of longstanding disease patients with recent onset RA (disease duration ≤ 1.5 year) as well as healthy controls (HC) were included between December 2010 and October 2011. Patients with diagnosis based on the American College of Rheumatology criteria for RA (14) were asked to participate. Exclusion criteria were diabetes mellitus (fasting blood glucose level >7 mmol/L, or use of antidiabetic drugs), pregnancy, renal impairment (serum creatinin >140 μmol/L), surgery in the past three months or a history of CVD. Healthy controls were recruited from hospital personnel and relatives from the researchers. Traditional cardiovascular risk factors such as smoking status, obesity (body mass index, BMI), hyperlipidemia and hypertension were recorded. Hypertension was defined as systolic arterial pressure above 140 mmHg and/or diastolic arterial pressure above 90 mmHg, or use of antihypertensive drugs prescribed with the aim to reduce blood pressure (15). Hyperlipidemia was diagnosed if plasma total cholesterol exceeded 6.21 mmol/l, plasma LDL cholesterol exceeded 3.36 mmol/l, plasma triglycerides exceeded 2.26 mmol/l, or current use of lipid-lowering drugs, similar to Brevetti et al. (15). Also the IMT (intima media thickness) of the carotid artery was measured by duplex ultrasonography at three locations, namely one centimeter proximal of the carotid bifurcation, on the bulbus carotis and one centimeter distal of the bulbus in the internal carotid artery, as a measure for degree of stenosis (16). The DREAM protocol was used for treatment of RA patients (17). In RA patients, disease activity was assessed using the Disease Activity Score for 28 joints (DAS-28 score) (18). The study was approved by the local medical ethics committee of the University Medical Center of Groningen and informed written consent was obtained from all participants.

Endothelial progenitor cells measured by Flow Cytometry analysis
Two hundred microliters of lithium-heparin blood was incubated with anti-CD34-PE (clone 5B1, IQproducts, Groningen, The Netherlands) and anti-CD133-APC (clone AC133, Miltenyi Biotec, Auburn, USA) or appropriate isotype controls. To lyse erythrocytes and fix remaining
cells, samples were incubated with FACS lysing solution (BD Bioscience) using a dilution of 1:10. PBS with 1% BSA was used to wash the cells. Flow cytometry measurements were performed on a BD Bioscience FACS Calibur flow cytometer, and analyzed using Winlist 6.0.

**Endothelial progenitor cell quantification by CFU-Hill assay**

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using lymphoprep (Axis Shield PoC As, Oslo, Norway). Subsequently, cells were washed with RPMI Medium 1640 (Lonza, Walkersville, MD, USA) + Gentamycin and suspended in culture medium consisting of RPMI supplemented with 20% v/v fetal calf serum (both from BioWhittaker, Verviers, Belgium), 2 mM L-glutamine (Gibco Products, Invitrogen, Breda, The Netherlands), 5 U/ml heparin (LEO Pharma, Ballerup, Denmark), 1% v/v PenStrep (Sigma, Zwijndrecht, The Netherlands), 50 μg/ml bovine brain extract (FBS, Gibco/Invitrogen, CA). The cells were plated on fibronectin-coated plates at 5 million cells per well and incubated at 37 °C and 5% CO₂. After 48 hours, non-adherent cells were harvested and diluted to a concentration of 1 million cells per well, and replated to a fibronectin-coated 24-well plate and cultured for 7 days. Medium was changed at day 3 and 6. CFUs were visually counted in quadruplo (4 wells) using an inversion microscope by two independent observers (NaJ, JW). The average of this count was used for statistical analysis.

**Quantification of endothelial activation markers by ELISA**

Serum levels of sVCAM-1, VEGF, ANGP-2 and TM were measured by ELISA according to the manufacturer's instructions (R&D Systems, Abingdon, UK). For determination of vWF we used an in house ELISA as previously described (19).

**Small Arterial Elasticity measurement**

In all patients and HC SAE was determined non-invasively using pulse-wave analysis measurement (CR-2000, Hypertension Diagnostics, Eagan, MN, USA), which is described previously (20).

**Intima Media Thickness**

The degree of stenosis of the carotid artery was measured at three locations, namely one centimeter proximal of the carotid bifurcation, on the bulbus carotis and one centimeter distal of the bulbus in the internal carotid artery by duplex ultrasonography using an Acuson 128XP ultrasound system with 7 MHz linear array transducers (Acuson Corp., Silicon Valley, California, USA) (21). A B-mode image was obtained after which a probe was positioned perpendicular to the far wall, showing an intima-media complex over approximately one centimeter. Mean IMT (the mean of the segment studied) and the maximum IMT (the highest IMT value found among the segment studied) were determined. As endpoint we used the mean of the mean (mean IMT) of the far wall IMT of the six imaged carotid
segments.

**Statistical analysis**

Values are presented as mean ± standard deviation or median (range) when appropriate, unless stated otherwise. The sample size of 29 patients was based on an earlier study, in which the sample size was calculated on the assumption that RA patients who achieved remission would normalize in SAE from 4.5 to 7.7 ml/mmHg100, based on a SD of 3.7 (6). For correlations, Pearson’s and Spearman’s correlation coefficients were used when appropriate. Non-paired continuous variables with a non-parametric distribution were analyzed using the Mann-Whitney U-test. The chi-square test was used for categorical variables and for small expected frequencies the Fischer’s exact test was used. To control for influence of differences in cardiovascular risk factors between patients and HC, multivariate linear regression analysis for correlations with EPC and CFU levels was performed by using forward inclusion of values of baseline characteristics with P values ≤0.10 in univariate analysis. The same was done to investigate the biomarker with the strongest association with EPC and CFU counts. A two-sided P value ≤ 0.05 was considered statistically significant. Statistical tests were done with the Statistical Package for the Social Sciences (SPSS statistics version 20.0, SPSS inc®, Chicago IL, USA).
Results

Patient characteristics
A total of 27 early RA patients, mean age 58 ± 9 years, and 15 HC, mean age 48 ± 13 years, were included. Of 29 included early RA patients, two patients were excluded; one due to a history of coronary artery disease (CAD) and one due to a myocardial infarction (MI). Traditional cardiovascular risk factors such as BMI, hyperlipidemia, hypertension and CRP of participants are shown in Table 1.

Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=27)</th>
<th>HC (n=15)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men, n (%)</td>
<td>6 (22)</td>
<td>6 (40.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Age, years</td>
<td>58 ± 9</td>
<td>48 ± 13</td>
<td>0.01</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.7 (19-37)</td>
<td>24 (20-33)</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>16 (59)</td>
<td>2 (13.3)</td>
<td>0.002</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>140.3 ± 24</td>
<td>126.9 ± 13</td>
<td>0.03</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>80.2 ± 11</td>
<td>70.5 ± 7</td>
<td>0.02</td>
</tr>
<tr>
<td>Dyslipidemia, n (%)</td>
<td>7 (25.9)</td>
<td>3 (21.4)</td>
<td>NS</td>
</tr>
<tr>
<td>LDL</td>
<td>3.6 ± 1</td>
<td>3.6 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>HDL</td>
<td>1.6 ± 0.4</td>
<td>1.7 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides mmol/L</td>
<td>1.1 ± 0.4</td>
<td>1.1 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.7 (4.7-6.6)</td>
<td>5.8 (4.6-6)</td>
<td>NS</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>5.0 (0.1-65)</td>
<td>1.1 (0.2-5.1)</td>
<td>0.03</td>
</tr>
<tr>
<td>DAS-28</td>
<td>2.4 (1.7-6.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>12 (11-16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RF, iU/mL</td>
<td>87 (8-1180)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive (&gt; 25 iU/mL), n (%)</td>
<td>17 (62.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CCP, U/mL</td>
<td>63 (1-340)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive (&gt; 10 U/mL), n (%)</td>
<td>22 (81.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No immunosuppressives, n (%)</td>
<td>4 (13.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methotrexate, n (%)</td>
<td>18 (66.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range), in users (mg/day)</td>
<td>15 (7.5–25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other immunosuppressive drugs, n (%)</td>
<td>6 (22.2)*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Three patients used Adalimumab, one patient was on Rituximab and two patients used prednisone. Hypertension was defined as: systolic blood pressure > 140mmHg, diastolic blood pressure > 90mmHg or use of antihypertensive drugs. Dyslipidemia was defined as: total cholesterol > 6.2 mmol/l, LDL > 3.2 mmol/l, triglycerides > 2.26 mmol/l or use of lipid lowering drugs. Unless otherwise indicated, data are expressed as mean ± standard deviation or median (range) when appropriate; percentages between brackets.
Medication use, presence of rheumatoid factor (RF), anti-CCP and DAS-28 score of RA patients are given as well. In the RA patients group, five patients were smokers and seven patients reported a history of smoking. In the HC group none of the participants reported smoking. Five RA patients used statins. In HC no history of cardiovascular disease was reported and no statins were used. The mean age of the RA patients was 10 years higher compared to the HC group. However, in univariate analysis age was not related to EPC counts (p=0.628). Age was not significantly related to CFU in univariate analysis (p=0.090), in contrast to smoking (p<0.001) and CRP (p=0.023). In multivariate analysis including age, smoking and CRP, only smoking was independently associated to CFU counts (p=0.002). No relation could be found between dosage of MTX, used in the majority of patients, and EPC numbers and CFU counts. The same yields for usage of other immunosuppressives and EPC numbers and CFU counts.

**Endothelial progenitor cells**

EPC numbers, determined by FACS analysis (CD34+/CD133+ cells), in RA patients were significantly lower compared to those in HC (481.4 ± 292.7 vs 715.4 ± 312.3, P=0.02, Figure 1A). The same applied for CFU counts (3.4 ± 2.3 vs 7.7 ± 1.9, P<0.0001, Figure 1B), suggesting impaired endothelial progenitor cell numbers in RA patients compared to HC. No correlation between EPCs and CFUs was found in RA (r=0.38, p=0.07) nor in HC (r=0.38, p=0.20, Figure 1C).

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**Figure 1.**

(A) CD34+/CD133+ cells of RA patients were significantly lower compared to EPC numbers of HC (p=0.02). (B) CFU counts were significantly lower in RA patients compared to healthy controls. (C) Correlation between CFU counts and EPC numbers.
Strikingly, a significant negative correlation was seen between RA disease activity DAS-28 and both EPC and CFU numbers ($r=-0.49$, $p=0.03$, Figure 2A and $r=-0.58$, $p=0.008$, Figure 2B respectively).

**Figure 2.**

![Graph A](image1) ![Graph B](image2)

Figure 2. EPC numbers compared to disease activity DAS-28 score in RA patients and endothelial activation markers in healthy controls and RA patients. (A) EPC numbers were negatively correlated to DAS-28 ($r=-0.49$, $p=0.03$) (B) CFU counts were negatively correlated to DAS-28 ($r=-0.58$, $p=0.008$)

### Endothelial activation markers

Measurement of sVCAM-1, VEGF, ANGP-2, TM and vWF levels showed that vWF, sVCAM-1 and VEGF were significantly increased in RA patients compared to HC ($192.2 \pm 75.8$ ng/ml vs $100.2 \pm 50.1$ ng/ml, $P=0.0002$, Figure 3A; $387.6 \pm 191.9$ ng/ml vs $293.6 \pm 49.8$ ng/ml, $P=0.05$, Figure 3B; and $149.9 \pm 84.6$ pg/ml vs $93.8 \pm 87.6$ pg/ml, $P=0.04$, Figure 3C respectively).

**Figure 3.**

![Graph A](image3) ![Graph B](image4) ![Graph C](image5)

Figure 3. (A) vWF is increased in RA patients compared to HC ($p=0.0002$) (B) sVCAM-1 is increased in RA patients compared to HC ($p=0.05$) (C) VEGF is increased in RA patients compared to HC ($p=0.04$).

ANGP-2 and TM levels were increased in RA patients compared to HC as well, but did not reach significance ($902.9 \pm 718.3$ pg/ml vs $759.5 \pm 497.5$ pg/ml and $3.9 \pm 1.3$ ng/ml vs $3.7 \pm 0.7$ ng/ml, respectively). Interestingly, significant negative correlations were found in RA patients, but not in HC, between sVCAM-1 levels and EPC numbers ($r=-0.58$, $p=0.004$) and ANGP-2 levels and EPC numbers ($r=-0.60$, $p=0.01$). In univariate analysis, sVCAM, ANGP-2 and SAE were related to EPC measurement, and vWF and SAE were correlated to CFU count.
(Table 2). Multivariate analysis showed that only sVCAM was independently associated with EPC (B: -1,456 (95% CI: -2635 - 0.277), p=0.017), while only SAE was independently associated with CFU counts (B: 5.193 (95% CI: 0.960 – 9.425), p=0.018).

**Table 2.** Association between EPC and CFU measurement, serum markers and SAE

<table>
<thead>
<tr>
<th></th>
<th>Univariate analysis</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>B (95% CI)</td>
<td></td>
</tr>
<tr>
<td><strong>A. relation with EPC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sVCAM-1, ng/ml</td>
<td>-1.561 (-2.840 -0.282)</td>
<td>0.018*</td>
</tr>
<tr>
<td>VEGF,</td>
<td>-1.186 (-3.154 0.782)</td>
<td>0.229</td>
</tr>
<tr>
<td>ANGPT-2, pg/ml</td>
<td>-0.269 (-0.484 -0.054)</td>
<td>0.016*</td>
</tr>
<tr>
<td>TM, ng/ml</td>
<td>42.468 (-145.322 230.257)</td>
<td>0.648</td>
</tr>
<tr>
<td>vWF, ng/ml</td>
<td>-0.754 (-2.514 1.006)</td>
<td>0.389</td>
</tr>
<tr>
<td>SAE, ml/mmHg</td>
<td>532.809 (16.737 1048.881)</td>
<td>0.043*</td>
</tr>
<tr>
<td><strong>B. relation with CFU</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sVCAM-1, ng/ml</td>
<td>-0.004 (-0.010 0.002)</td>
<td>0.151</td>
</tr>
<tr>
<td>VEGF,</td>
<td>-0.004 (-0.014 0.007)</td>
<td>0.518</td>
</tr>
<tr>
<td>ANGPT-2, pg/ml</td>
<td>-0.001 (-0.002 0.001)</td>
<td>0.462</td>
</tr>
<tr>
<td>TM, ng/ml</td>
<td>0.522 (-0.316 1.361)</td>
<td>0.215</td>
</tr>
<tr>
<td>vWF, ng/ml</td>
<td>-0.014 (-0.025 -0.002)</td>
<td>0.019*</td>
</tr>
<tr>
<td>SAE, ml/mmHg</td>
<td>4.682 (0.580 8.783)</td>
<td>0.026*</td>
</tr>
</tbody>
</table>

Multivariate regression analysis was performed by using markers with P values ≤0.10* in univariate analysis, to elucidate the biomarker with the strongest association with EPC and CFU counts. SAE was log transformed.
Small artery elasticity

SAE was decreased in RA patients compared to HC, as shown in Figure 4A (P=0.02), indicating increased stiffness of arteries in RA patients. Strikingly, SAE was significantly correlated to EPC numbers (Figure 4B, r=0.40, p=0.02) and to CFU counts (r=0.35, p=0.04). However, when RA patients and HC were evaluated separately, no significant correlation remained (EPC numbers, Figure 4C).

Figure 4.

Figure 4. Vascular measurements in RA patients and healthy controls. (A) Small Arterial Elasticity was significantly reduced in RA patients (4.0 ± 1.5 ml/mmHg) compared to healthy controls (6.9 ± 3.8 ml/mmHg). (B) SAE was positively correlated to EPC counts (r=0.40, p=0.02). (C) No significant correlation remained when RA patients and HC were evaluated separately.

Intima Media Thickness

IMT measurements showed no significant difference in the degree of stenosis of healthy controls and RA patients (p=0.19, 0.74 ± 0.2 vs 0.98 ± 0.5 mm respectively). No correlation was found between IMT and neither EPC nor CFU measurement.

Discussion

The current study shows that circulating EPC and CFU counts, as well as SAE, are reduced in early RA patients compared to healthy controls, while endothelial activation markers vWF, sVCAM-1 and VEGF were increased. Furthermore, EPC and CFU counts were correlated to SAE and inversely related to endothelial activation markers, such as VCAM-1, ANGP-2, and to disease activity. These data suggest that decreased numbers of EPCs in peripheral blood might play a role in accelerated development of atherosclerosis in RA patients. The results remained significant after correcting for the differences in baseline characteristics between both groups in prevalence of pre-existing cardiovascular risk factors and age (Table 1). Controversy still exists about the exact definition, identity and phenotype of EPCs. Many different types of blood cells and endothelial cells are being included in the term EPC. Although cells co-expressing CD34⁺, VEGFR2 (KDR) and CD133⁺ are commonly considered to be EPCs (22), additional markers such as CD45, flk-1, Tie-2 and CD146 are considered markers of EPCs as well. To test the population of KDR⁺/CD34⁺/CD133⁺ cells, a minimum
amount of events is needed to identify an adequate number of this scarce population (<0.01% of PBMCs without enrichment) using flow cytometry. Two hundred microliters of peripheral blood was not enough to detect these triple marked cells. As adding KDR to the analysis of cells increases specificity of analysis, this can be seen as a limitation of our study (22). To improve the detection sensitivity, we identified EPCs as CD34+/CD133+ (23,24).

Some experts consider broadening the definition of an EPC to: “hematopoietic lineage cells that display proangiogenic properties” (25). Given the controversy mentioned we deliberately chose to include the spindle like cells that grow in a CFU-Hill to determine EPCs as well. One reason for the lack of correlation between the two methods could be the possibility that the different methods sample EPCs at different stages of differentiation (26). Furthermore, new insights show CFU-Hill reflect a variety of blood cells including monocytes, lymphocytes and hematopoietic progenitor cells skewed towards the myeloid lineage, and therefore express different cell types as EPCs measured by flowcytometry. The fact that CFU-Hill fails to exclude the presence of hematopoietic cells in the colonies and do not directly demonstrate vessel forming ability is a disadvantage of this method (25).

The observation that disease activity in RA patients, measured by DAS-28, shows a correlation with EPC and CFU counts has been demonstrated before (27). However, in the latter study RA patients with mean disease duration of 10 years were included, while we included early RA patients (with a disease duration ≤ 1.5 year). By doing so, long term influences of medication were reduced to a minimum. To our knowledge, our study is the first to show that EPC and CFU counts are associated with endothelial activation, namely, ANGP-2 and sVCAM-1 levels were inversely correlated to EPC numbers. In a retrospective study we previously demonstrated increased levels of VEGF and ANGP-2 in a large group of recent-onset RA patients (28). These markers were shown to be highly correlated to inflammation and disease activity, whereas RA patients who developed cardiovascular disease had significantly higher ANGP-2 levels than those who did not.

As mentioned, negative correlations were found in RA patients between sVCAM-1 levels and EPC numbers. Also, sVCAM-1 levels were higher in RA patients compared to HC, and independently associated with EPC numbers. sVCAM-1 induces firm adhesion of inflammatory cells at the vascular surface and contributes to endothelial dysfunction, reflected by SAE, which is a major factor in cardiovascular disease. As SAE was independently associated with CFU counts, this indicates that sVCAM-1 might be a good marker for predicting cardiovascular risk in chronic inflammatory diseases such as rheumatoid arthritis (29).

Previous research has shown that RA patients with active disease suffer earlier from cardiovascular disease (30) and that EPC levels are decreased in RA patients (27), as well as in systemic lupus erythematosus (SLE) patients (31,32), which is in concordance with our findings. A possible explanation for the lower EPC numbers in inflammatory diseases could be that these cells migrate to other tissues, for instance to the inflamed synovial membrane. This hypothesis is supported by the work of Rüger et al. who found an enrichment of EPCs.
in the inflamed joint (33). In a collagen-induced arthritis model Silverman et al. showed selective recruitment of EPCs to inflamed joint tissue, demonstrating a role for the VCAM-1/VLA-4 (very late activation antigen) system in directing EPCs to synovial fibroblasts (34). In a recent paper Isozaki et al. showed that CXCL16 and its receptor CXCR6 are a central ligand receptor pair that induce EPC recruitment and blood vessel formation in the RA joint (35).

An alternative explanation could be that EPC numbers are decreased due to continuous endothelial damage, as occurs in patients with muscular dystrophy. In this disease, the satellite cells, a type of progenitor cells in skeletal muscle, get exhausted. Furthermore, cells that do remain in these patients show evidence of accelerated ageing (36). Decrease in EPCs might also be due to use of immunosuppressive drugs. However, no relation could be found between dosage of MTX, used in the majority of patients, and EPC numbers. The observation of rising EPCs numbers when disease activity decreases, strongly argues against suppressive effects of anti-rheumatic drugs. Nevertheless, further research to examine the exact role of anti-rheumatic drugs on EPC numbers is necessary.

In contrast to our findings, De Villeroché et al. indicated that EPC levels are increased in RA patients due to disease activity. They suggested that this is due to different factors, including inflammation, vascular injury, and potentially the immune response with bone marrow changes preceding inflammation in the synovium (37). However, the mean disease duration in this study was sixteen years, a different subpopulation of EPCs was used and patients with conventional cardiovascular risk factors were excluded, so these data cannot be compared to the present study. The same applies to the study of Rodríguez-Carrio et al. who found increased EPCs in SLE patients, but used another subpopulation of EPCs (mature EPCs (CD34+VEGFR2+) and ECs (VEGFR2+)) (38).

It is of interest to investigate whether the decrease in EPC numbers is typical for inflammatory diseases with cardiovascular involvement, or whether it also occurs in other diseases. The group of Kim et al. investigated the number of circulating EPCs in gastric and breast cancer patients. Although the plasma levels of VEGF (mobilization of EPCs from the bone marrow) were elevated, there was no increase in EPC number (39). However, in a study with type two diabetes patients, a generalized decrease in progenitor cell populations was observed. This reduction was, consistent with our study, negatively associated with disease severity (40) and suggests EPCs are lowered in various diseases with cardiovascular involvement. Patients with diabetes were excluded to participate in the study, to prevent a bias in EPC measurement as a result of the disease. Previous work showed that a reduction in EPC number could be a consequence of ageing (41). In our study, the mean age in the patient group was 10 years higher compared to the HC group. No significant association with age could be found. However, the number of study participants is limited for subgroup analyses, which is a major limitation to this study. As EPCs are important in endothelial repair and are related to endothelial activation markers and disease activity, but not to established atherosclerosis (measured by IMT), their reduced numbers in RA patients are in
agreement with increased cardiovascular mortality in these patients. Furthermore, these data suggest that EPC count might serve as a very early biomarker in the atherosclerotic process in subjects with rheumatoid arthritis.

Conclusions

CFU counts and EPC numbers are reduced in early RA patients, are inversely related to RA disease activity, and correlate to endothelial activation markers and endothelial dysfunction. Measurement of endothelial progenitor cells by flowcytometry or CFU culture might be of value in cardiovascular risk assessment in RA patients (25) as EPC counts may represent a balance between the magnitude of injury and the capacity for endothelial cell repair (12). Still, it is not clear whether reduced EPC numbers directly contribute to the pathophysiology of atherosclerosis, and whether the decreased numbers found in early RA patients is a consequence of influx in synovial tissue in RA or results from medication use. To answer these questions further studies, including assessment of functionality of EPCs in RA patients and their link with clinical events, are needed.
References


