Endothelial dysfunction in patients with rheumatoid arthritis is associated with a reduced number and impaired function of endothelial progenitor cells

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Abstract

Objective: Rheumatoid arthritis (RA) is associated with increased morbidity and mortality attributable to accelerated atherosclerosis and cardiovascular events. Endothelial progenitor cells (EPC) seem to play an important role in the defence system against arteriosclerosis.

Methods: We studied number and function of endothelial progenitor cells in young RA patients (n=13) with low disease activity (DAS 28 3.5±0.3) and healthy control subjects (n=13). Endothelial function was investigated by agonist-induced endothelium dependent vasodilation measured by the forearm blood flow technique. Migratory activity and adhesion of EPC to TNFα activated mature endothelial cells and components of the extracellular matrix were tested in vitro. Putative precursor populations (CD34+, CD34+/CD133+ and CD34+/KDR+ hematopoietic stem cells) were measured by flow cytometric analysis.

Results: Acetylcholine-induced, endothelium-dependent vasodilation was reduced by approximately 50% in RA patients indicating endothelial dysfunction, while endothelium-independent vasodilation in response to glyceryl-trinitrate was at control level. We found significantly reduced numbers of EPC in these patients compared to control. Migratory activity of EPC was decreased in RA patients. Adhesion to mature endothelial cells after activation with TNF-α was enhanced only in control. The adhesion to matrix proteins and the number of putative precursor cell lineages was comparable in both groups.

Conclusion: This study demonstrates that endothelial dysfunction in RA patients with low-grade inflammation is associated with a reduced number and partial dysfunction of EPC. Further studies have to explore whether interventions that potentially ameliorate the number and function of EPC also result in an improvement of endothelial function in these patients.
Introduction

Rheumatoid arthritis (RA) is associated with increased morbidity and mortality attributable to accelerated atherosclerosis and cardiovascular events [1, 2]. We and others have recently demonstrated endothelial dysfunction, an early sign of atherosclerosis, even in young RA patients with low-grade inflammatory activity [3, 4]. Among diseases associated with an increased cardiovascular risk RA is unique because conventional risk factors (e.g. hypertension, diabetes, hyperlipidemia) cannot be assumed to play the major causative role in this context. It has been proposed that in RA increased levels of circulating inflammatory mediators may cause activation and damage of endothelial cells which contributes to endothelial dysfunction [5]. Very recent studies have shed new light on the mechanisms of endothelial repair. It has been shown that bone marrow-derived endothelial progenitor cells (EPC) are involved in the maintenance of the endothelial cell layer [6]. Hill et al. reported that the number of endothelial progenitor cells may be a surrogate biological marker for vascular function and cumulative cardiovascular risk [7]. In their study the number of EPC was strongly correlated with the Framingham score and the endothelial function of young healthy male volunteers. It has been shown that patients at risk for coronary artery disease have decreased numbers of circulating EPC with impaired activity [8]. The authors suggested a significant role of EPC in patients with coronary artery disease.

It is conceivable that an altered number and/or function of EPC contribute to the accelerated arteriosclerosis seen in RA patients. Hypothetically, chronic inflammation or deficiency of growth factors required for maturation and differentiation may cause putative “EPC-dysfunction” in RA. Therefore it might be potentially relevant for the pathogenesis and later therapeutic endeavours to study whether EPC release from bone marrow and/or functions of ex vivo expanded EPC are altered in RA patients.

Methods

Characteristics of patients and controls

The study was approved by the institutional review board of our medical center. All participants gave written informed consent. Thirteen patients with documented RA were selected from the outpatient program of the division of rheumatology. RA was known for 7.7±2.7 years. All underwent routine clinical examination. To avoid confounding factors potentially relevant for EPC kinetics or measurement of endothelial dysfunction we excluded patients with diabetes mellitus a past medical history of coronary artery disease and smokers. None of the patients had been treated with statins previously. Matched healthy volunteers served as control group. Characterization of the study population is shown in table 1. Disease-modifying anti-rheumatic drugs included methotrexate (MTX) in all and anti-TNF alpha antibodies in six patients. None of the patients received glucocorticoids. Disease activity of RA was well controlled as demonstrated by a low disease activity score (DAS 28).

Forearm blood flow analysis

Studies were performed in a quiet, temperature controlled room (23-25°C) with the subjects resting supine. At the beginning of each experimental session a clinical evaluation was performed and venous blood was collected for measurement of laboratory parameters, isolation of EPC and flow cytometric analysis. The Disease Activity Score 28 tender joint count was assessed in all patients [9].

Studies were performed as described previously [3]. Briefly, infusions of test agents were given into the brachial artery of the non-dominant arm via a 27 G needle. Forearm blood flow (FBF) was measured in both arms by venous occlusion plethysmography. During all
recording periods the hands were excluded from the circulation by a wrist cuff inflated to a suprasystolic pressure of 220 mmHg.

Measurements of baseline FBF were started 20 min after arterial puncture. Each dose of agent was given over 5 min at a constant rate of 1 mL/min. Between the infusions of different agents we kept a 30 minute control period during which isotonic saline was infused. The study protocol consisted of infusions of graded doses of acetylcholine (ACH; Miochol-E®, Ciba Vision, Germering, Germany; 55, 110 and 220 nmol/min) and glyceryl-trinitrate (GTN; Perlinganit®, Schwarz Pharma, Monheim, Germany; 2.2, 4.4 and 8.8 nmol/min).

EPC Culture Assay
Mononuclear cells (MNCs) were isolated by density-gradient centrifugation with Histopaque-1077 (Sigma) from 27 ml of peripheral blood. Immediately after isolation 1x10⁶ mononuclear cells were plated on an 8-chamber culture glass slide coated with fibronectin (Sigma) and were maintained in endothelial basal medium (EBM, CellSystems) supplemented with EGM SingleQuots and 5% FCS. After 3 days in culture, nonadherent cells were removed; adherent cells underwent cytochemical analysis on day 4.

Characterization of EPC
To detect the uptake of 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine-labeled acetylated LDL (DiLDL) (Molecular Probes), cultivated cells were incubated with DiLDL (10µg/mL) at 37°C for 1 hour. Thereafter cells were fixed with 2% paraformaldehyde for 10 minutes and incubated with FITC-labeled UEA I (lectin, 10µg/mL; Sigma) for 1 hour. Cells staining positive for both lectin and DiLDL were judged to be EPC. Their numbers were counted per well by two blinded investigators. They counted 3 to 5 randomly selected high power fields (hpf) per well. To confirm the phenotype, the expression of surface marker proteins was measured by flow cytometric analysis as recently reported by us [10].

Flow cytometric measurement and analysis
Flow cytometric measurement was performed on a FACS Calibur flow cytometer (Becton Dickinson).
First, a three color analysis was performed using CD45 FITC (DAKO Cytomation), CD34 PE (BD Sciences), and CD133 APC (Miltenyi Biotec). After the incubation of peripheral blood with the above mentioned antibodies (15 min at room temperature) red cells were lysed for 10 min with FACS lysing solution (BD Sciences). Thereafter, cells were washed and resuspended in 500µl PBS (Seromed). Measurement was performed after setting a live gate on cells with low side scatter in the dot plot SSC vs. CD34. In this gate 50,000 events were registered.
Second, on cultured EPC two color analyses using VE Cadherin FITC (BenderMedSystems), CD31 PE (Cymbus Biotechnology) and CD146 FITC (Biocytex) were performed. At least 10,000 cells were measured after gating cells in a FSC/SSC dot plot. Fluorescence intensity was analysed using GeoMean.
Third, EPC were characterized with KDR (ReliaTech) after indirect staining using a PE conjugated goat anti-mouse secondary antibody (DAKO Cytomation). Non-matched, isotype specific antibodies served as controls in each measurement.

Migration Assay
Isolated EPC were detached mechanically, harvested by centrifugation, resuspended in 300µl EBM (without VEGF) and counted. 2x10⁵ EPC were placed in the upper chamber of a modified Boyden chamber. The chamber was placed in a 24-well culture dish containing EBM and human recombinant VEGF (50ng/mL) (Sigma). After 24 hours of incubation at 37°C, the lower side of the chamber, containing the migrated cells, was washed with PBS and
fixed with 2% paraformaldehyde. For quantification cell nuclei were stained with DAPI and counted in 3 random microscopic fields by 2 blinded investigators. Measurement was performed in a duplicate manner.

To address the effect of microinflammatory environment on EPC bioactivity we cultured EPC from control subjects (n=6) EGM-2 containing 20% human serum pooled from 6 healthy volunteers or from 6 RA patients (instead of 5% FCS, standard cell culture condition). Thereafter the migration assay was performed as described above.

**EPC Adhesion to Matrix Molecules**
Collagen Typ IV (100µg/mL), Fibronectin (100µg/mL) or Laminin (2µg/mL) was coated onto 4-chamber culture glass slides for 2 hours at 37°C. Wells were blocked with 1% BSA in PBS for 1 hour and 1x10^5 EPC were added to each well. Non adherent cells were removed after 1 hour. Adherent cells were counted in 3 random microscopic fields by 2 blinded investigators.

**EPC Adhesion to Endothelial Cells**
A monolayer of human coronary artery endothelial cells (HCAEC) was prepared 72 hours (in 4-chamber glass slides) before 1x10^5 EPC (DiLDL labeled) were added to each well and incubated for 2 hours at 37°C. HCAEC were pretreated for 12 hours with TNFα (1ng/mL) or medium. Nonattached cells were gently removed. Cells were fixed with 2% paraformaldehyde for 10 minutes. Nuclei of all cells were stained by DAPI. EPC and HCAEC were counted in 3 hpf by two blinded investigators. Percentage of EPC of the total cells per hpf was used for statistical analysis.

**Apoptosis assay**
Quantitative determination of cells undergoing apoptosis was determined using an Annexin V apoptosis detection kit (Alexis) according to the manufacturer’s instructions. Percentage of apoptotic cells was measured in EPC (originated from healthy volunteers; n=5) cultured with and without MTX (0.1µM, 1µM, 10µM and 100µM) for 4 days. Cells were stained with annexin V-FITC and propidium iodide (PI) subjected to flow cytometric analysis. To exclude necrotic cells only annexin positive cells were counted. Data are given as mean±SEM in percentage of annexin V^+/PI^− cells (representative of apoptotic cells).

**Blood levels of VEGF and Interleukin-6**
Blood levels of VEGF and IL-6 were measured by highly-sensitive ELISA assay (R&D Systems) according to the manufacturer’s instructions. Samples were checked by serial dilution and measurements were performed in duplicate.

**Calculation and Statistics**

**Forearm blood flow studies**
In general each determination of FBF was calculated as the mean of the last 5 individual FBF measurements. Results are presented as absolute FBF in the infused and in the non-infused arm. For statistical analysis of the vascular responses to ACH and GTN we compared the results obtained in the infused arm by two-way ANOVA for repeated measurements. The comparison of baseline FBF between groups was made by Student’s t-test. For correlation analyses we calculated an endothelial function index (EFI) as follows:
EFI (%) = \[\text{FBF (ACh 220 nmol/min) – FBF (baseline before ACh)} / \text{FBF (GTN 8.8 nmol/min) – FBF (baseline before GTN)} \times 100 – 100.\]

The EFI gives the endothelium-dependent vasodilation in percent of the endothelium-independent vasodilation. Equal values of both types of vasodilation result in an EFI of zero.

**EPC studies**

Comparison of continuous variables was performed using two-way ANOVA. Statistical significance was considered to be present at the 5% level. All statistical analyses were performed using the computer software SPSS for Windows 12.0. All data are given as mean±SEM.

**Results**

**Forearm blood flow analysis**

Endothelial function of the study participants was tested by agonist-induced endothelium dependent vasodilation as measured by the forearm blood flow technique. Acetylcholine-induced vasodilation was significantly reduced in RA patients (figure 1A) while endothelium-independent vasodilation studied by infusion of glyceryl-trinitrate was at control level (figure 1B).

**EPC characterization**

EPC were characterized as cells dual-stained positive for DiLDL and UEA I, more than 95 percentage of the adherend cells were positive for both (figure 2A). In addition, their endothelial phenotype was confirmed by demonstrating the expression of the endothelial marker proteins vascular endothelium-cadherin (VE-cadherin), CD31 and KDR by flow cytometric analysis after 4 days of culture. (figure 2B-D). We found that 45.9±5.4% were positive for VE-cadherin, 90.9±5.9% for KDR and 96.9±1.5% for CD31. Human coronary artery cells served as positive control cells (VE-cadherin: 69.8±6.9%; KDR: 59.7±7.7%; CD31:99.6±0.2%)

It is important to exclude relevant numbers of circulating mature endothelial cells contributing to the observed outgrow of EPC from the starting MNC population. CD146 is a marker for mature endothelial cells, our cultured cells stained positive for the endothelial markers mentioned above and negative for CD146. Therefore it is likely that the studied cells are progenitor cells but not circulating mature endothelial cells (figure 2E).

**EPC number**

MNCs from healthy volunteers and patients were cultured for 4 days and EPC were characterized and counted as described above. The number of EPC in the culture assay was significantly reduced in RA patients (230±29 vs. 490±48 EPC/hpf; P<0.001), representative fluorescent microscopy pictures are shown in figure 3.

We found a positive correlation between the number of EPC and the vascular response expressed by the endothelial function index over the entire study population (figure 3D).

**EPC function in vitro**

We observed a partially dysfunction of EPC in RA patients, in detail: migratory activity in response to VEGF was reduced in the RA patients (50.2±7.4 vs. 88.5±8.5 EPC per hpf; P<0.005, figure 4A). If MNC of control subjects (n=6) were cultured in EGM-2 supplemented with pooled serum of RA patients (n=6) the EPC showed a decreased migratory activity
compared with MNC cultured in EGM-2 supplemented with serum of healthy volunteers (39.8±5.1 vs. 61.4±5.9 cells/hpf; P=0.02). Results are shown in figure 4B.

The adhesion of EPC to non-activated HCAEC cultured from MNCs of patients was comparable with the number of adherent cells of the control group (9.7±1.3 vs. 9.2±1.8 in % of total cell number/hpf, n=13). We observed a significant increase in adherent EPC of 88% in control subjects after activation of HCAEC with TNFα (P<0.005). EPC from RA patients showed only a mild increase of 20% after TNFα activation, (P=0.5). The effect of TNFα activation was proofed in six participants in each group. There were no differences between patients and control subjects in the adhesion of EPC to matrix proteins. The highest adherence in both groups was observed when EPC were added to fibronectin coated chamber slides (control: 19.7±1.6/hpf and RA patients: 19.8±2.9/hpf), followed by adhesion to laminin (control: 15.8±1.5/hpf and RA patients: 15.5±3.3/hpf). Adhesion to collagen type IV coated chamber slides was found at a level of 13.2±1.0/hpf in control subjects and 11.6±1.2/hpf in patients.

**Number of CD34⁺, CD34⁺/KDR⁺, CD34⁺/CD133⁺ cells**

Some of putative precursor populations of EPC (CD34⁺, CD34⁺/KDR⁺, CD34⁺/CD133⁺ cells) were investigated by flow cytometric analysis. We found reduced numbers of each studied lineage in RA patients but the differences did not reach statistical significance. The following data were obtained: CD34⁺ cells: 0.020±0.003% in RA patients vs. 0.027±0.004% in control (P=0.25); CD34⁺/CD133⁺ cells: 0.015±0.002% vs. 0.022±0.004% (P=0.13); CD34⁺/KDR⁺ cells: 0.005±0.001% vs. 0.007±0.002% (P=0.5).

**Role of MTX induced EPC apoptosis**

To evaluate the potential influence of MTX on apoptosis we analyzed the binding of annexin V in EPC from healthy volunteers cultured for 4 days with and without MTX. A significant elevated apoptosis rate was found after incubation of EPC with 10µM MTX and 100µM MTX compared to cells cultured in MTX free environment (control: 15.7±1.9; 0.1µM: 15.7±3.0; 1µM: 16.5±2.2; 10µM: 21.5±3.4; 100µM: 22.5±3.4 in % of total cells, P<0.01).

**Cytokines**

Mean blood levels of VEGF were similar in both groups (VEGF: 406.8±34.6 pg/mL in control subjects vs. 324.3±27.5 pg/mL in RA patients. IL-6 was nearly 2.5-fold elevated in RA patients (1.4±0.3 vs. 3.3±0.5 pg/mL, P<0.003). The IL-6 blood levels negatively correlated with the EPC number in vitro when correlation was calculated over the entire study population (figure 5). No such correlation was found between EPC number and hsCRP.

**Discussion**

This study was designed to test number and function of EPC in young patients with low grade activity of RA on standard MTX treatment and proven endothelial dysfunction. The latter was demonstrated by an impairment of agonist-induced endothelium dependent vasodilation in this particular group of patients compared to matched control subjects. In the culture assay we found the total number of EPC to be significantly reduced in RA patients. This result adds to previous findings showing a reduced EPC number in patients with diseases associated with an increased risk for cardiovascular events, e.g. diabetes mellitus type 1 and 2, coronary artery disease, hyperlipidemia and chronic renal insufficiency. Moreover, we found a significant correlation between EPC number and endothelial function over the entire study population. The count of putative precursor populations (CD34⁺, CD34⁺/CD133⁺, CD34⁺/KDR⁺ cells) detected by flow cytometric analysis was reduced in RA-patients but the differences did not
reach statistical significance. The ability to express endothelial marker proteins after ex vivo expansion is also reported for CD34 negative cells, CD14+, CD14+/VEGFR2+, CD14+/Tie-2+ cells and mesenchymal stem cells are able to develop a endothelial phenotype in vitro [13, 14, 15, 16, 17]. Trans-differentiation of monocytes positive for VEGFR2 has been reported, too [18, 19]. Confirming recent data reported by Kuwana et al. our finding may indicate that stem cell lineages other than CD34+ stem cells contribute to the generation of EPC [20]. Differences in the cell populations mentioned above or an altered transdifferentiation of monocytes may therefore have contributed to the observed reduced number of EPC in RA patients in vitro.

In addition to the endothelial progenitor cell count we also investigated the state of important functional features of EPC in vitro. We found a significantly reduced migratory activity of EPC in RA patients compared to control while the ability of EPC to adhere to mature endothelial cells and to components of the extracellular matrix was mainly at control level. Activation of mature endothelial cells with TNF-α caused an increased adhesion of EPC originated from healthy subjects but not of EPC from RA patients. These results are compatible with partial EPC dysfunction in RA patients on standard MTX treatment.

In principal there are two likely explanations for the alterations in EPC number and function observed in RA patients: first, the intake of disease-modifying anti-rheumatic drugs and second, the presence of a steady low-grade inflammation.

We were therefore interested whether our findings could be explained by MTX - induced apoptosis of EPC. Our in vitro measurements of apoptotic EPC cultured in MTX containing medium demonstrated a small albeit significantly increased apoptosis rate compared to control medium. Although this finding is unlikely to entirely explain the reduced EPC number in RA patients, this mechanism could be a contributing factor. In this context it would be interesting to study the influence of MTX on hematopoietic bone marrow stem cells, too.

High sensitive C-reactive protein and IL-6 were elevated in the serum of RA patients indicating the presence of low-grade inflammatory activity. A relationship between the number of circulating angiogenic cells and the inflammatory activity in RA patients has been recently reported by Grisar et al. They found an inverse correlation between the disease activity assessed by the DAS 28 and CD34/CD133/KDR positive cells in the peripheral blood [21]. We are unable to confirm this particular result. However, our finding of a negative correlation between the IL-6 level and the EPC number over the entire study population may support the hypothesis that the individual microinflammatory state is related to the number of circulating EPC. Furthermore, it is known from in vitro experiments that C-reactive protein attenuates EPC survival and very recently it has been demonstrated that C-reactive protein has the potential to decrease the angiogenic function of EPC [22, 23].

A further mechanism involved in the reduced number of EPC in the circulation could be homing of circulating EPC to sites of active neo-vascularization and inflammation. Rüger et al. reported recently that endothelial progenitor cells are present in the synovial membranes of RA patients, where they formed cell cultures to generate new vessels [24].

Summarized, our data show a reduced number and an impaired function of endothelial progenitor cells in young RA patients with low disease activity and proven endothelial dysfunction. Further studies have to explore whether interventions that potentially ameliorate the number and function of EPC also result in an improvement of endothelial function in these patients.

Competing interests: None declared
Ethics approval
The study was approved by the institutional review board of our medical center. All participants gave written informed consent.

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Figure Legends

Figure 1: Forearm blood flow (FBF) in response to acetylcholine and glyceryl-trinitrate in control subjects and RA patients; * P<0.003 by ANOVA for repeated measurements.

Figure 2: A, Over 95% of adherent cells are positive for DiLDL and UEA-I (upper right field) B, The phenotype of cultured cells was studied by flow cytometric analysis of specific marker proteins: CD31, VE-cadherin and KDR (for endothelial differentiation) and CD146 (mature endothelial cells) was used for exclusion of contaminating adult EC. Representative images are shown. Plots show specific antibody staining (black) and isotype controls.

Figure 3: Dual-stained cells positive for both lectin (green) and DiLDL (red) were judged as EPC and counted per well. Representative high power fields of a control subject (A) and a RA patient (B) are shown. C, Number of EPC in RA patients and controls, P<0.001. D, Positive correlation between EPC count in vitro and endothelial function estimated by endothelial function index in the entire study population.

Figure 4: A, Migration of EPC from RA patients and controls. B, Migratory activity of EPC from control subjects after culture in EGM-2 supplemented with pooled serum of healthy volunteers (white) or serum of RA patients (black). * P<0.005; ** P=0.02

Figure 5: The EPC count measured in vitro by the culture assay showed an inverse correlation with blood levels of IL-6 in the entire study population.
References

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Characterization of RA patients and healthy control subjects.

Data are means ± SEM. * P < 0.003; # P < 0.05
Figures

Figure 1

**Infused arm**

- Acetylcholine [nmol/min]
- FBH [mL/dL x min]
- RA (n=13) vs. C (n=13)

**Control arm**

- Acetylcholine [nmol/min]
- FBH [mL/dL x min]
- RA (n=13) vs. C (n=13)

**Infused arm**

- Glyceryl-trinitrate [nmol/min]
- FBH [mL/dL x min]
- RA (n=13) vs. C (n=13)

**Control arm**

- Glyceryl-trinitrate [nmol/min]
- FBH [mL/dL x min]
- RA (n=13) vs. C (n=13)
Figure 4

A

B

EPC/high power field

RA (n=13)  C (n=13)

EPC/high power field

RA  C

*  **
Figure 5

![Graph showing the relationship between Interleukin-6 [pg/mL] and EPC number/HPF, with a negative correlation coefficient $R = -0.564$ and statistical significance $P < 0.01$.](image-url)