Depletion of Endothelial Progenitor Cells in the Peripheral Blood of Patients With Rheumatoid Arthritis

Johannes Grisar, MD; Daniel Aletaha, MD; Carl W. Steiner, CT; Theresa Kapral, MD; Sabine Steiner, MD; Daniela Seidinger, MT; Günter Weigel, MD; Ilse Schwarzinger, MD; Wolfgang Wolozcszuk, PhD; Günter Steiner, PhD; Josef S. Smolen, MD

Background—Rheumatoid arthritis (RA) is characterized by increased cardiovascular morbidity and mortality that cannot be explained solely by traditional cardiovascular risk factors. Cardiovascular morbidity is related to disease activity and can be normalized by effective therapy. Because the quantity of endothelial progenitor cells (EPCs) in the peripheral blood is correlated inversely with cardiovascular risk, we studied whether such abnormalities could also be observed in patients with RA.

Methods and Results—EPCs were determined in 52 RA patients and in 16 healthy referents (HRs) by fluorescence-activated cell-sorting (FACS) analysis. Patients were divided into groups characterized by active disease (n=36) and low disease activity (n=16). Cells that were positive by flow cytometry for CD34/KDR/AC133 within the lymphocyte population were characterized as EPCs. Furthermore, in subgroups of patients, circulating EPCs were also quantified by a colony-forming-unit (CFU) and a circulating angiogenic cell (CAC) assay. EPCs were significantly decreased in RA patients suffering from active disease compared with those from HRs, as measured by FACS analysis (0.026±0.002% versus 0.045±0.008%, respectively, P<0.05), CFU assay (mean of 5±2 versus 18±5 CFU/well in HRs, P<0.05), and CAC assay (mean of 7±2 versus 52±16 positive cells/high-power field, P<0.005). In contrast, the frequency of circulating EPCs from patients with low disease activity was comparable to that of healthy individuals (0.052±0.006% by FACS analysis), CFU assay (10±5 CFU/well), and CAC assay (mean of 25±5 positive cells). Moreover, EPC quantities in peripheral blood were correlated inversely with disease activity as assessed by the disease activity score (r=−0.38, P<0.01).

Conclusions—Our observations indicate that active RA is associated with a depletion of circulating EPCs. This might be one of several factors contributing to the increased cardiovascular risk in RA. (Circulation. 2005;111:204-211.)

Key Words: progenitor cells ■ rheumatoid arthritis ■ angiogenesis ■ cardiovascular diseases ■ antibodies
preexisting endothelial cells/angioblasts (angiogenesis) or by peripheral recruitment of endothelial progenitor cells (EPCs) (vasculogenesis). EPCs, primarily described in the landmark observation of Asahara et al., represent a population of bone marrow–derived, CD34–, VEGF receptor-2 (VEGFR-2 or kinase-insert domain receptor [KDR])–, AC133–positive cells that have the ability to differentiate into endothelial cells and thus, make a significant contribution to new blood vessel formation. It has long been thought that EPCs could be cultured from peripheral blood mononuclear cells (PBMCs) on fibronectin and then express receptors for LDL and lectin. However, recent observations indicate that these cultured cells are mainly monocyte/leukocyte-derived entities; nevertheless, they also contribute to angiogenesis by secreting proangiogenic factors and are now referred to as circulating angiogenic cells (CACs). The number of circulating EPCs and CACs has been described as being inversely correlated with several risk factors for coronary artery disease and to constitute a surrogate marker for various aspects of vascular function. On the basis of all these observations, we hypothesized that there might be an alteration of circulating EPCs and CACs in RA.

Methods

Characteristics of Study Patients and HR Subjects

EPCs were studied by FACS analysis from 52 random patients suffering from RA according to 1987 American College of Rheumatology criteria. Significant hypertension or diabetes was the exclusion criterion. Patients were subgrouped by disease activity into a group with active disease, reflected by a disease activity score using 28 joint counts (DAS28) ≥ 3.2 (n = 36) and a group with low disease activity or inactive disease (DAS < 3.2, n = 16). Disease activity variables, such as joint counts and global assessments, were evaluated by an independent assessor. Sixteen healthy volunteers (some of the authors and nonmedical persons known to the authors who had neither a history of ischemic cardiovascular events, inflammatory disease, or autoimmune disease nor any other chronic disease) were selected to be matched by age and sex and served as the healthy reference (HR) population. The characteristics of patients and HRs are summarized in the Table. The local ethics committee approved the study, and patients and HRs provided informed consent for this research. Surrogates of inflammatory response (erythrocyte sedimentation rate and CRP), rheumatoid factor, and erythropoietin (Epo) were determined according to standard procedures.

As shown in the Table, at the time of the study in the active-RA group, 2 patients were not receiving any DMARDs, 20 were receiving methotrexate (MTX), 5 were receiving leflunomide, 2 were receiving salazopyrine, and 3 patients were being treated with a combination of DMARDs; 4 patients were receiving subcutaneous or intravenous TNF-blocker therapy (etanercept or infliximab with MTX). Twenty-three of the patients were also being treated with low-dose prednisolone (≤ 10 mg/d).

Flow Cytometry Analysis

Fluorescence-activated cell sorting (FACS) analyses of EPCs were performed as described by Vasa and coworkers. In brief, 100 μL of peripheral blood was incubated with a biotinylated monoclonal antibody against human KDR (Becton Dickinson), followed by staining with streptavidin–Quantum Red conjugate (Sigma). Samples were then incubated with fluorescein isothiocyanate (FITC)–conjugated CD34 (BD Pharmingen) and phycoerythrin-conjugated AC133 phycoerythrin (Miltenyi) antibodies. Control stainings were performed with isotype-matched antibodies. Incubation was followed by lysis and fixation with BD lysing solution. Acquisition was then performed on a Becton Dickinson FACS flow cytometer (FACScan) and included 100 000 to 300 000 events per sample. Cells positive for CD34/KDR/AC133 within the lymphocyte population were characterized as EPCs. These analyses were performed for all 52 patients and 16 HRs.

CFU Assay

Circulating EPCs were also quantified by the colony-forming unit (CFU) assay as described by Hill et al in randomly selected, active-disease (n = 10) and inactive-disease (n = 9) RA patients and in 10 HRs. For this purpose, 5 × 10⁶ PBMCs obtained by density gradient centrifugation on Ficoll-Paque (Amersham Biosciences) were plated on fibronectin-precoated, 6-well plates and cultivated in growth medium 199 (Gibco) containing 20% fetal calf serum and penicillin (100 U/mL)/streptomycin (100 μg/mL, Gibco) for 48 hours. Nonadherent cells were then recollected, and 10⁶ cells/well were replated onto fibronectin-coated, 24-wells plates (Nunc) and cultured in duplicate samples for 7 days in growth medium that was changed every 3 days. CFUs, characterized by a central cluster surrounded by emerging cells, were then counted. Confirmation of endothelial cell lineage derivation of the CFUs was performed by immunostaining with biotinylated anti-KDR, followed by streptavidin–Quantum Red and FITC-conjugated CD34.

CAC Culture Assay

In another subgroup comprising 9 active- and 6 inactive-RA patients as well as 6 HRs, CACs, known to be derived mainly from the monocyte/leukocyte lineage and attributed to proangiogenic effects, were quantified by culture assay. In brief, PBMCs were isolated by density gradient centrifugation on Ficoll-Paque. PBMCs (4 × 10⁶/well) were plated on 24-well culture plates (Nunc) coated with fibronectin in 1 mL endothelial basal medium-2 supplemented with endothelial growth medium-2 single quots (both from Cambrex Bio Science) containing fetal bovine serum, hydrocortisone, human VEGF-A, human PGE-B, human epidermal growth factor, insulin-like growth factor-1, and ascorbic acid in appropriate amounts. After 4 days, nonadherent cells were removed by washing twice with phosphate-buffered saline, and adherent cells were again incubated in 1 mL medium. After 3 more days, EPCs were analyzed by incubating them with 1, 1'-dioctadecyl-3,3',3',3'-tetramethylindocarbocyanine perchlorate–labeled, acetylated LDL (acLDL, BioMedical Technologies) for 3 hours and subsequent fixation with 4% paraformaldehyde for 20 minutes. Cells were then incubated for 1 hour with FITC-labeled Ulex europaeus agglutinin I (lectin, 10 μg/mL, Sigma). Three randomly selected, high-power fields were...
evaluated by fluorescence or confocal microscopy. Only cells with double-positive staining for acLDL and lectin were counted as CACs. To exclude the possibility that the results had been influenced by changes in monocyte counts, we also performed differential blood counts for each donor. To confirm CAC derivation from the monocyte/leukocyte lineage, expression of CD11a, CD11b, CD14, and CD45 within the CAC population was measured by FACS analysis.

Enzyme-Linked Immunosorbent Assays
VEGF, bFGF, TNF, TNF receptors 1 and 2 (TNFR-I and TNFR-II), and Epo were quantified in the sera of 31 to 40 patients with RA and in 11 to 16 HRs with the use of commercial ELISA kits (TNF, Biosources; all others, R&D Systems). The minimum levels of detection were as follows: 5.0 pg/mL (VEGF), 0.22 pg/mL (bFGF), 3.0 pg/mL (TNF), 0.77 pg/mL (TNFR-I), 0.6 pg/mL (TNFR-II), and 0.6 mU/mL (Epo).

Statistical Evaluation
All data are presented as mean±SEM, unless stated otherwise. Statistical evaluations were performed with SPSS for Windows, version 8.0 (SPSS Inc). Data were evaluated with a normality test, equal variance test, and Student’s t test. Data not normally distributed were analyzed by nonparametric methods. When >2 groups were compared, 1-way ANOVA was used (given equality of variances). Post hoc Bonferroni-adjusted group comparisons were performed when appropriate. A probability value <0.05 was considered statistically significant.

Results
CD34/KDR/AC133-Positive Cells Are Decreased in Active RA
When PBMCs were analyzed by flow cytometry for the presence of EPCs, CD34/KDR/AC133-positive cells were found to be significantly decreased in patients with active RA compared with matched HRs (Figure 1A, 0.045±0.008% in the HR group versus 0.026±0.002% in RA patients, P<0.05). The frequency of circulating EPCs in patients with low disease activity or inactive disease (0.052±0.006%) was comparable to that of HRs but differed significantly from that of active-RA patients (P<0.002). In fact, EPC levels in peripheral blood were correlated inversely with disease activity as assessed by the DAS28 (r=-0.38, P<0.01, Figure 1B). When the DAS28 subcomponents were analyzed further, EPC levels were correlated inversely with swollen (r=-0.39, P<0.01) and tender (r=-0.45, P<0.005) joint counts, but not with ESR and the global assessment.

Effects of DMARDs: Patients Treated With Anti-TNF Show Normal EPC Frequencies
Subanalyses performed of active-RA patients treated with different DMARDs suggested that as long as the disease was active, therapy with traditional DMARDs had no impact on EPC levels (Figure 2A). Patients treated with MTX or those receiving either no DMARD or DMARDs other than MTX showed EPC quantities close to the mean of all active-RA patients (stated earlier). In contrast, among patients with DAS <3.2, regardless of therapy, circulating EPC proportions were similar to those in HRs (Figure 2A). However, there was one exception: Among patients with active disease who were receiving TNF-blocker therapies, EPC levels (0.045±0.007%) were comparable to those of HRs and patients with low disease activity (Figure 2A), even though their DAS scores were similar to those of all other patients with active disease (5.57±0.81 versus 5.04±0.19). As shown in Figure 2B, patients on low-dose steroid treatment did not differ from patients without steroids in regard to their EPC levels (P=0.93).

CFU Assays Confirm a Reduction of EPCs in RA
When the CFUs (Figure 3A and 3B, which shows CFUs by inverted microscopy) were counted for HRs and for patients with active disease (DAS28>3.2) and low disease activity (DAS28<3.2), the flow cytometric observations of EPC depletion in active RA were confirmed: Mean CFU counts were significantly lower in patients with active RA (5±2/
well) when compared with HR (18±5/well, P<0.05). In contrast, CFU counts among patients with low disease activity were twice as high (14±4/well) as those of patients with active RA and statistically did not differ from the counts of HRs (P=0.95, Figure 3C).

CAC Assays Indicate Decreased Angiogenic Potential of CD14-Positive Cells in RA

Cultivation of EPCs from a group of 9 RA patients with a DAS28 ≥3.2, from 6 patients with a DAS28 <3.2, and from 6 HRs again resulted in a significantly reduced number of EPCs in the cultures of active-RA patients. As shown in Figure 4, after 7 days in culture, cells positive for lectin and acLDL, representing CACs, were found to be reduced by almost 90% compared with those from HRs (mean of 52±16 versus 7±2 acLDL/lectin-positive cells per high-power field, P<0.005). In patients with low disease activity, the mean of positive cells detected in culture assays was 25±5, which was not significantly different from that of HRs (P=0.45, Figure 4). In Figure 5, representative confocal fluorescence microscopy images of double staining for lectin and acLDL are shown. Relative monocyte counts in the differential blood counts, which could have influenced the results in the CAC assay, indicated no significant differences among the 3 groups (7.7±0.5% in HRs, 7.8±1.2% in inactive RA patients, and 6.5±0.5% in active RA patients). FACS analysis of CD11a, CD11b, and CD14 as well as CD45 expression confirmed that the majority of CACs was of leukocyte/monocyte origin (data not shown).

Enzyme-Linked Immunosorbent Assays

bFGF serum levels were significantly increased among active-RA patients (9.7±1.0 pg/mL) when compared with HRs (3.6±1.3 pg/mL, P<0.0005) but were not detectable in inactive-RA patients. Likewise, VEGF levels were much higher among patients with active RA (629±69 pg/mL, P<0.0005). In the groups of active- and inactive-disease patients as well as in the HR group, bFGF and VEGF levels showed no correlation with EPC levels. Furthermore, serum Epo levels were significantly increased in the group of active-RA patients (22±5 mU/mL) compared with patients with low DAS (37.5±10.1 pg/mL, P<0.0005). In the group of active RA, treatment with low-dose steroid (prednisolone ≥10 mg/d) did not affect EPC levels (0.026±0.004% in both groups, n=23 with and n=13 without [w/o] steroid treatment, P=0.93). All other abbreviations are as defined in text.

Figure 2. Lack of effect of DMARD and glucocorticoid therapy on EPC levels, except for TNF blockade. A, In group of active-RA patients (frequency of EPCs in total cell population, 0.026±0.002%, n=36), those treated with TNF blockers (0.045±0.007%, n=4) showed EPC frequency comparable to HRs (n=16) and to patients with low disease activity (n=16, see Figure 1A). MTX (n=20 in active RA, n=10 in inactive RA) or other DMARDs (n=12 in active RA and n=6 in inactive RA) had no effect on circulating EPCs in active disease (ANOVA P<0.005). B, In active-RA, treatment with low-dose steroid (prednisolone ≥10 mg/d) did not affect EPC levels (0.026±0.004% in both groups, n=23 with and n=13 without [w/o] steroid treatment, P=0.93). All other abbreviations are as defined in text.
In the group of active-RA patients, levels of both soluble TNF receptors showed a significant correlation with CRP (TNFR-I, \( r = 0.434, P = 0.02 \); TNFR-II, \( r = 0.387, P = 0.05 \)), but there was no relation with EPC levels.

**Discussion**

Our data demonstrate a depletion of peripheral EPCs in patients with active RA. This observation was consistent, whether EPCs were determined by flow cytometry (CD34+/KDR/AC133-positive cells), CFU assay, or CAC assay. Moreover, EPC levels were inversely related to disease activity. In contrast, inactive RA was not associated with a significant reduction of EPC frequencies compared with those in HRs. However, this conclusion may be limited by relatively low power because of the small sample size.

Because RA patients with active disease have a high propensity to suffer from cardiovascular disorders, especially myocardial infarction,\(^{34}\) and because EPC levels are correlated inversely with cardiovascular risk factors,\(^{26}\) our findings support these associations. Importantly, patients with low disease activity had normal EPC levels, in line with indications of reduced cardiovascular mortality among patients treated effectively with DMARDs\(^{7}\) and with the very recent finding of EPC inhibition by CRP.\(^{35}\) Interestingly, whereas Epo has been shown to mobilize EPCs,\(^{36,37}\) circulating EPCs were low in RA patients, despite increased Epo concentrations, and there was no correlation between Epo and EPC levels; this may be partly related to Epo resistance, as

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**Figure 3.** CFUs of EPCs. A and B, CFUs after 9 days in culture were characterized by central cluster surrounded by emerging cells. Representative images from one HR are shown. (A, \( \times 200 \) magnification; B, \( \times 100 \) magnification). C, Quantification of CFUs confirmed significant differences between HR, active RA, and inactive RA as tested by ANOVA (\( P < 0.05 \)): in active RA (\( n = 10 \)), numbers of CFUs were significantly lower when compared with HRs (5±2 CFU/well vs 18±5 CFU/well, \( n = 10, P < 0.05 \) after Bonferroni adjustment), confirming results of EPC counts by FACS. Patients with inactive RA had intermediate counts (10±5 CFU/well). No significant alterations were observed when comparing active with inactive RA (\( P = 0.44 \) after adjustment) and HR with inactive patients (\( P = 0.95 \) after adjustment). Abbreviations are as defined in text.

**Figure 4.** CACs are depleted in active RA. ANOVA showed significant influence of RA on CACs (\( P < 0.005 \)). CAC staining in culture assays by acLDL and lectin and evaluation by confocal or fluorescence microscopy indicated that active RA is associated with depletion of EPCs (mean of 7±2 cells/high-power field [\( n = 9 \)] vs 52±16 cells/high-power field in HR [\( n = 6 \), \( P < 0.005 \) after Bonferroni adjustment]). Quantity of positive cells from inactive-RA patients (mean of 25±5 cells/high-power field, \( n = 9 \)) did not differ from that of either active-RA patients (\( P = 0.15 \) after adjustment) or HRs (\( P = 0.45 \) after adjustment). Abbreviations are as defined in text.

pg/mL, \( P < 0.01 \); TNFR-II, 4150±113 vs 3633±145 pg/mL, \( P < 0.02 \). In the group of active-RA patients, levels of both soluble TNF receptors showed a significant correlation with CRP (TNFR-I, \( r = 0.434, P < 0.02 \); TNFR-II, \( r = 0.387, P < 0.05 \)), but there was no relation with EPC levels.
previously described in RA. However, Epo levels were correlated with CRP.

None of the patients studied had known cardiovascular abnormalities. The percentage of persons taking hydroxymethylglutaryl coenzyme A reductase inhibitors (statins), which are known to increase EPC numbers, did not differ between HRs and RA patients. Moreover, serological markers of angiogenesis were increased among active-RA patients, as reflected by the high bFGF levels; VEGF was only mildly elevated. As with Epo, the observation that VEGF and bFGF increase EPC mobilization and proliferation does not appear to be reflected in patients with active RA. Thus, in RA, the levels of factors known to influence EPC mobilization or angiogenesis are elevated in relation to disease activity, but apparently their effects do not translate to the cellular level of EPCs. The observed decrease of VEGF and the low levels of TNF and bFGF in inactive-RA patients likely result from effective treatment leading to a normalization of their production. Among RA patients with high serum levels of TNF, circulating EPC levels were significantly decreased, suggesting a possible TNF dependence of the phenomenon observed in our study. This is further supported by the observation that EPC levels were within the normal range in patients treated with TNF blockers despite having high disease activity. It is also interesting in this context that a recent study reported a significant decrease of heart failure in RA patients treated with TNF blockers when compared with other therapies.

Figure 5. CACs in culture. CACs, stained by acLDL (upper panel), lectin (middle panel), and both acLDL and lectin (lower panel) after 7 days of culture in confocal microscopy images, from HRs (A), inactive-RA patients (B), and active-RA patients (C). Cell counts indicate that EPC quantity was affected by disease and amount of disease activity. Representative images are shown. Magnification ×400. Abbreviations are as defined in text.
In contrast to RA, patients with gastric or breast cancer have numbers of circulating EPCs similar to those of HRs and have high VEGF levels; although similar to the findings reported here, patients suffering from another chronic disease associated with increased cardiovascular risk, diabetes melitus type 1 and type 2, are significantly deficient in EPCs compared with healthy individuals. In diabetes, serum TNF levels were increased among patients (n=14) with high (≥15 pg/mL) TNF levels (0.040±0.005% vs 0.023±0.003%, P<0.05). Abbreviations are as defined in text.

In summary, the results of our study may open a new view: The decrease of EPCs and CACs in active RA might constitute an important event associated with the increased cardiovascular risk in RA patients. This risk as well as the decrease in EPCs may be primarily related to the increased TNF production known to be pathogenic in RA. In fact, also in this study, TNF levels were increased among patients with active RA and were correlated with surrogates of disease activity as well as low EPC frequencies. Consequently, effective therapy leading to low disease activity, as well as blockade of TNF, may lead to normalization of EPC levels. Thus, the mechanism by which effective DMARD therapy as well as TNF blockade may normalize the cardiovascular

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