Number and Migratory Activity of Circulating Endothelial Progenitor Cells Inversely Correlate With Risk Factors for Coronary Artery Disease

Mariuca Vasa, Stephan Fichtlscherer, Alexandra Aicher, Klaudia Adler, Carmen Urbich, Hans Martin, Andreas M. Zeiher, Stefanie Dimmeler

Abstract—Recent studies provide increasing evidence that postnatal neovascularization involves bone marrow–derived circulating endothelial progenitor cells (EPCs). The regulation of EPCs in patients with coronary artery disease (CAD) is unclear at present. Therefore, we determined the number and functional activity of EPCs in 45 patients with CAD and 15 healthy volunteers. The numbers of isolated EPCs and circulating CD34/kinase insert domain receptor (KDR)-positive precursor cells were significantly reduced in patients with CAD by \(-40\%\) and \(-48\\%\), respectively. To determine the influence of atherosclerotic risk factors, a risk factor score including age, sex, hypertension, diabetes, smoking, positive family history of CAD, and LDL cholesterol levels was used. The number of risk factors was significantly correlated with a reduction of EPC levels \((R=-0.394, P=0.002)\) and CD34-/KDR-positive cells \((R=-0.537, P<0.001)\). Analysis of the individual risk factors demonstrated that smokers had significantly reduced levels of EPCs \((P<0.001)\) and CD34-/KDR-positive cells \((P=0.003)\). Moreover, a positive family history of CAD was associated with reduced CD34-/KDR-positive cells \((P=0.011)\). Most importantly, EPCs isolated from patients with CAD also revealed an impaired migratory response, which was inversely correlated with the number of risk factors \((R=-0.484, P=0.002)\). By multivariate analysis, hypertension was identified as a major independent predictor for impaired EPC migration \((P=0.043)\). The present study demonstrates that patients with CAD revealed reduced levels and functional impairment of EPCs, which correlated with risk factors for CAD. Given the important role of EPCs for neovascularization of ischemic tissue, the decrease of EPC numbers and activity may contribute to impaired vascularization in patients with CAD. The full text of this article is available at http://www.circresaha.org.

Key Words: coronary disease ■ angiogenesis ■ endothelium

Recent studies provide increasing evidence that postnatal neovascularization does not exclusively rely on sprouting of preexisting vessels, but involves bone marrow–derived circulating endothelial progenitor cells (EPCs). These bone marrow–derived endothelial progenitor cells (EPCs) are considered to originate from hematopoietic stem cells, which are positive for CD34 or the more immature marker protein CD133.1–4 In animals, CD34-positive leukocytes were shown to home to sites of ischemia, to express endothelial antigens such as kinase insert domain receptor (KDR) (vascular endothelial growth factor [VEGF] receptor-2),2,5 and to make a significant contribution to adult blood vessel formation.6 Importantly, injection of isolated CD34-positive cells or cultivated EPCs enhances neovascularization6–8 and accelerates the restoration of blood flow in diabetic mice.9 Moreover, increased neovascularization by bone marrow–derived angioblasts or CD34-positive cells was shown to improve cardiac function.10,11

The regulation of EPC mobilization and differentiation in patients with coronary artery disease (CAD) has not been studied so far. Therefore, we investigated the influence of atherosclerotic risk factors on the number and functional activity of EPCs. The present study demonstrates that patients with CAD exhibit reduced levels and functional impairment of EPCs. The reduction of the levels and migratory capacity of EPCs were inversely correlated with the number of risk factors. Multivariate analysis of the individual risk factors revealed smoking as the major independent predictor for the reduction of EPC levels, whereas the migration of EPCs was mainly influenced by hypertension.

Materials and Methods

Characteristics of Study Patients and Healthy Control Subjects

Forty-five patients with angiographically documented CAD were prospectively studied. The patient characteristics are summarized in
TABLE 1. Baseline Clinical Characteristics of the Patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n=45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>62.4±1.7</td>
</tr>
<tr>
<td>Male sex, No. (%)</td>
<td>34 (75.6)</td>
</tr>
<tr>
<td>No. of diseased coronary arteries (%)</td>
<td>14 (31.1)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>28 (62.2)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>10 (22.2)</td>
</tr>
<tr>
<td>Current smoking</td>
<td>16 (35.6)</td>
</tr>
<tr>
<td>Family history of CAD</td>
<td>20 (44.4)</td>
</tr>
<tr>
<td>Left ventricular ejection fraction, %</td>
<td>53.8±1.5</td>
</tr>
<tr>
<td>Current medication, No. (%)</td>
<td>39 (86.7)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>7 (15.6)</td>
</tr>
<tr>
<td>Coumarins</td>
<td>38 (84.4)</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>40 (88.9)</td>
</tr>
<tr>
<td>Insulin</td>
<td>6 (13.3)</td>
</tr>
<tr>
<td>Lipid profile, mg/dL</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>219±6.6</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>143.9±5.9</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>45.3±1.5</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>149±11</td>
</tr>
</tbody>
</table>

Data are mean±SE.  ACE indicates angiotensin-converting enzyme.

Table 1. Patients with concomitant inflammatory or malignant disease were excluded. Patients had stable CAD (n=22), acute coronary syndrome (n=9), or myocardial infarction (n=14) with positive troponin test. In patients with myocardial infarction, blood samples were taken 4.2±0.4 days after the acute ischemic event. None of the patients had previously been treated with a statin. The LDL cholesterol serum levels ranged from 57 to 261 mg/dL at the time of inclusion into the study.

The age-matched healthy control group (n=9) consisted of 3 women and 6 men with a mean age of 60±5 years without any evidence of CAD by history and physical examination. Moreover, 6 healthy volunteers (5 women and 1 men; mean age 29±2.3 years) were included in the study. Informed consent was obtained from all patients and healthy volunteers; the study protocol was approved by the local Ethics Committee of the University of Frankfurt.

Definition of Risk Factors for CAD

To determine the overall risk factor load of an individual subject, a risk factor score including age >40 years, sex, hypertension, diabetes, smoking, positive family history of CAD, and hypercholesterolemia was calculated according to Vita et al. Hypercholesterolemia was defined as a history of hypertension for >1 year that required the initiation of antihypertensive therapy by the primary physician. Smoking was defined as patients revealing a history of smoking for >2 pack-years and current smoking. Positive family history for CAD was defined as documented evidence of CAD in a parent or sibling before 60 years of age. Hypercholesterolemia was defined as fasting LDL cholesterol levels exceeding 130 mg/dL. Diabetes was defined as the need for oral antidiabetic drug therapy or insulin use.

Isolation, Cultivation, and Characterization of EPCs

Mononuclear cells were isolated by density gradient centrifugation with Biocoll (Biochrom, Berlin, Germany) from 20 mL of peripheral blood, and 4×10^6 mononuclear cells were plated on 24-well culture dishes coated with human fibronectin and gelatin (Sigma) in endothelial basal medium (EBM) (CellSystems) supplemented with endothelial growth medium SingleQuots and 20% FCS. After 4 days in culture, nonadherent cells were removed by thorough washing with PBS and adherent cells underwent cytotoxic analysis. To detect the uptake of 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine–labeled acetylated LDL (DiLDL), cells were incubated with DiLDL (2.4 μg/mL) at 37°C for 1 hour. Cells were then fixed with 2% paraformaldehyde and analyzed with FITC-labeled Ulex europaeus agglutinin I (lectin, 10 μg/mL) (Sigma) for 1 hour. Dual-staining cells positive for both lectin and DiLDL were judged as EPCs and counted per well. Two or three independent investigators evaluated the number of EPCs per well by counting 3 randomly selected high-power fields. The reproducibility of the method was tested in healthy volunteers, which revealed essentially unchanged levels during a 4-week observational period (data not shown).

To confirm the endothelial phenotype, the expression of endothelial marker proteins was additionally measured by flow cytometry. EPCs were detached with 1 mmol/L EDTA in PBS, and cells were incubated for 15 minutes with mouse anti-human KDR (Sigma), anti–vascular endothelium cadherin (Santa Cruz), or anti–von Willebrand factor (vWF; Becton Dickinson). Phycocerythrin (PE)–conjugated goat anti-mouse F(ab')2, DAKO was used as secondary antibody. For DiLDL/von Willebrand double staining, EPCs were incubated with DiLDL as described above, washed with PBS, and then stained with rabbit anti-human vWF (Calbiochem-Novabiochem) followed by swine anti-rabbit FITC antibodies (DAKO). All incubations were performed at 4°C followed by fixation in 2% paraformaldehyde before fluorescence-activated cell sorter analysis. Single- and 2-color flow cytometric analyses were performed using a FACSscan flow cytometer (Becton Dickinson).

To further characterize the endothelial phenotype of EPCs, shear stress–induced upregulation of endothelial NO synthase (eNOS), a specific feature of differentiated endothelial cells, was assessed as previously described.

Flow Cytometry Analysis

A volume of 100 μL peripheral blood was incubated for 15 minutes in the dark with monoclonal antibodies against human KDR (Sigma) followed by PE-conjugated secondary antibody, with the FITC-labeled monoclonal antibodies against human CD45 (Becton Dickinson), with the PE-conjugated monoclonal antibody against human CD133 (Milteny), and with FITC- or PE-conjugated monoclonal antibodies against human CD34 (Becton Dickinson). Isotypic-identical antibodies served as controls (Becton Dickinson). After incubation, cells were lysed, washed with PBS, and fixed in 2% paraformaldehyde before analysis. Each analysis included 60,000 events.

Migration Assay

Isolated EPCs were detached using 1 mmol/L EDTA in PBS (pH 7.4), harvested by centrifugation, resuspended in 500 μL EBM, and counted, and 2×10^5 EPCs 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 (50 ng/mL). After 24 hours incubation at 37°C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cell nuclei were stained with DAPI. Cells migrating into the lower chamber were counted manually in 3 random microscopic fields.

Statistical Analysis

Data are expressed as mean±SEM. Continuous variables were tested for normal distribution with the Kolmogorov-Smirnov test and compared by means of 1-way ANOVA. In case of nonnormal distribution, nonparametric tests were used (Mann-Whitney U test or Kruskal-Wallis ANOVA on ranks). The number of risk factors (risk factor score) was considered a continuous variable. Categorical
variables were compared by means of the χ² test and the Fisher exact test. Linear regression analysis and nonparametric bivariate correlation (Spearman rank correlation coefficient) were used to compare the number and migratory activity of EPCs with each individual risk factor as well as with the risk factor score, respectively. The interaction between risk factors and EPC number and migratory activity was examined by multivariate analysis using the multiple stepwise logistic regression model. Statistical significance was assumed if a null hypothesis could be rejected at $P<0.05$. All statistical analysis was performed using SPSS for Windows 7.0.

Results

Influence of Risk Factors on EPC Numbers

To determine the influence of risk factors on EPC levels, mononuclear cells were isolated from 45 patients with CAD (for patient characteristics see Table 1) and 15 healthy volunteers. Adherent EPCs were characterized by DiLDL uptake and concomitant lectin binding (Figure 1A). The endothelial origin was further documented by demonstrating the expression of KDR (79±7.8%), vWF (74±9%), and vascular endothelium cadherin (80±8%) by flow cytometry (Figure 1B). Moreover, EPCs were shown to be positive for both DiLDL uptake and vWF (Figure 1C). Moreover, shear stress–induced eNOS upregulation, a specific feature of differentiated endothelial cells, was further demonstrated by immunoblotting (Figure 1D).

As illustrated in Figure 2A, the number of EPCs was significantly reduced by ≈40% compared with age-matched healthy volunteers. The number of risk factors was significantly correlated with a reduction of EPC levels (Figure 2B). Moreover, an inverse correlation between EPC levels and the number of risk factors was also demonstrated when only patients with CAD were included into the analysis ($n=45$, $R=-0.301$, $P=0.045$). Univariate analysis of the individual risk factors (Figures 2C through 2E) revealed that smoking was associated with significantly lower EPC levels, whereas a minor but nonsignificant reduction of EPC levels was detected in the presence of hypertension, diabetes, and a positive family history of CAD (Figure 2C). Neither age nor LDL cholesterol levels were correlated with the number of EPCs (Figures 2D and 2E). By multivariate analysis, only smoking remained as an independent predictor of reduced EPC numbers (Table 2).

Influence of Risk Factors on Circulating CD34-/KDR-Positive Progenitor Cells

Circulating EPCs are considered to be characterized by expression of CD34 and the VEGF receptor KDR. Therefore, we
directly determined the number of CD34/KDR double-positive cells in the peripheral blood of a subset of 35 patients with CAD by flow cytometry (Figure 3). CD34-/KDR-positive cells were significantly reduced by \( \approx 48\% \) in patients with CAD compared with 9 age-matched healthy volunteers (Figure 4A). The number of risk factors was inversely correlated with the levels of CD34-/KDR-positive cells \((R = 0.537, P < 0.001; \text{Figure 4B})\). Similar results were obtained when only patients with CAD were included \((R = 0.356, P = 0.036, n = 35)\). Univariate analysis identified smoking and a positive family history of CAD as significant predictors of reduced CD34/KDR levels (Figure 4C). In addition, increased age and elevated LDL cholesterol serum levels significantly correlated with lower numbers of CD34-/KDR-positive cells \((R = 0.124, P = 0.392, n = 35)\). Multivariate analysis revealed that smoking is the most important independent predictor of reduced circulating CD34-/KDR-positive cells, whereas a positive family history of CAD, age, and LDL cholesterol levels did not reach statistical significance (Table 2).

**Risk Factors Do Not Influence CD34- or CD133-Positive Hematopoietic Precursor Cell Numbers**

To investigate whether the overall number of hematopoietic precursor cells is affected in patients with CAD, the total number of CD34-positive cells was determined. However, CD34-positive leukocytes were similar in patients with CAD compared with age-matched volunteers \((0.056 \pm 0.005\% \text{ versus } 0.05 \pm 0.007\% \text{ CD34/CD45-positive cells, respectively})\). Moreover, the number of the more immature hematopoietic cells expressing CD133 was not different in patients with CAD \((0.085 \pm 0.009\% \text{ versus } 0.087 \pm 0.01\% \text{ in healthy volunteers})\). A potential influence of risk factors on CD34- or CD133-positive cells was further evaluated by correlation with the risk factors for CAD. However, neither CD34 nor CD133 or CD34/CD133 double-positive cells were correlated with the number of risk factors \((R = 0.279, P = 0.55; R = 0.120, P = 0.406; \text{and } R = 0.124, P = 0.392, \text{respectively})\).

**Effect of Risk Factors on Migratory Capacity of EPCs**

To assess the functional activity of EPCs, migration of isolated EPC in response to VEGF was determined in 28 patients with CAD using a modified Boyden chamber. As illustrated in Figure 5A, the migratory capacity of EPCs isolated from patients with CAD was significantly impaired compared with healthy age-matched volunteers. The number of risk factors for CAD significantly correlated with a reduction of the migratory capacity of EPCs (Figure 5B). To investigate whether the impaired migratory activity of EPCs to VEGF might be due to a downregulation of the VEGF receptor, the expression of KDR was analyzed in isolated EPCs by flow cytometry (see Figure 1B). However, the
expression of the KDR receptor on the EPCs was not correlated with the numbers of risk factors ($R = 0.117$, $P = 0.667$; $n = 16$).

Analysis of the individual risk factors revealed that EPC migration was inhibited in patients with hypertension (Figure 5C). Moreover, a significant negative correlation was detectable with respect to age ($R = -0.515$, $P = 0.001$) and LDL cholesterol levels ($R = -0.452$, $P = 0.005$) (Figures 4D and 4E), whereas individuals with a positive family history of CAD exhibited a minor but nonsignificant reduction of their EPC migration (Figure 5C). In contrast, gender, smoking, and diabetes did not have a significant effect on EPC migration (Figure 5C). When we included all risk factors in a multivariate analysis, only hypertension was associated with a significant reduction of EPC migration (Table 2).

**Discussion**

The results of the present study demonstrate that atherosclerotic risk factors inversely correlate with the number of differentiated EPCs and CD34-/KDR-positive circulating progenitor cells. Moreover, the functional activity of isolated EPCs as measured by their migratory capacity was impaired in relation to the number of risk factors. Analysis of the individual risk factors indicated that smoking is a major factor, which contributes to reduced numbers of circulating EPCs. In contrast, the migratory capacity appears to be mainly influenced by hypertension, but independent of smoking. Serum LDL cholesterol levels, age, and a positive family history of CAD were additionally shown to determine the number of circulating CD34-/KDR-positive cells and EPC migration. However, no influence of LDL cholesterol levels, age, or a positive family history of CAD was detected when the EPCs were counted after isolation and cultivation. One may speculate that the ex vivo cultivation procedure ameliorates the influence of the risk factors on the number of EPCs, whereas the direct measurement of CD34-/KDR-positive cells might more closely resemble the in vivo conditions.

Given that several experimental studies indicate a significant contribution of EPCs for adult neovascularization,6–8 the reduction in the number of EPCs and their functional impairment might contribute to reduced vascularization in patients with CAD. Indeed, previous studies indicated that atherosclerotic risk factors can impair the formation of new blood vessels in response to tissue ischemia. Thus, increases in age or hypercholesterolemia are associated with reduced angiogenesis.15,16 Moreover, clinical data suggest a relation between impaired coronary blood flow regulation and risk
factors for CAD. Strikingly, the risk factors identified in the present study to affect the number and functional activity of EPCs are well established to also impair the function of mature endothelial cells. Age, hypertension, smoking, cholesterol levels, and a positive family history of CAD, as well as the overall number of risk factors, have all been shown to be associated with impaired endothelium-mediated vasodilator function of the coronary circulation. Therefore, one may speculate that the impairment of circulating EPCs may contribute to an insufficient regeneration of the endothelium, which may lead to endothelial dysfunction. In support of this hypothesis may be recent experimental data demonstrating that the age-associated decrease of endothelial function correlates with enhanced endothelial cell apoptosis. However, because the role of EPCs for regeneration of the endothelium in mature vessels is not yet clarified, further studies are necessary to address this hypothesis.

The mechanisms by which risk factors for CAD reduce EPC numbers remain to be determined. Because the overall population of CD34 or CD133 hematopoietic progenitor cells was not altered, the present data suggest a specific effect of risk factors on the CD34+/KDR-positive subpopulation of hematopoietic cells, which are considered as endothelial precursor cells. There are several possible scenarios by which atherosclerotic risk factors could reduce the number of circulating EPCs. One explanation might be increased apoptosis of mature progenitor cells. Indeed, CD34-positive EPCs were shown to be very sensitive to apoptosis induction. Moreover, risk factors for CAD such as smoking are known to increase oxidative stress, a well-established stimulus for apoptotic cell death. Alternatively, risk factors may interfere with the signaling pathways regulating EPC differentiation or mobilization. It is known that cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) or VEGF can mobilize EPCs. However, in the present study, the number of risk factors did not correlate with systemic VEGF or GM-CSF serum levels (data not shown).

In addition to the reduction of EPC numbers, EPCs isolated from patients with CAD exhibited a decreased migration in response to VEGF, indicating a functional impairment. The reduced response to VEGF might be due to a downregulation of the VEGF receptor KDR, which mediates the VEGF effects in endothelial cells. However, at least the expression of the KDR receptor was not reduced in EPCs derived from patients with CAD compared with those from healthy volunteers (data not shown) and was not correlated to the number of risk factors, suggesting that defects in the downstream signaling pathways might be responsible for the impairment of cell migration. Of the individual risk factors investigated, hypertension emerged as the most important independent predictor of a dramatically reduced EPC migration. These data are consistent with the observation of structural alterations of the microvascular bed in hypertension. Moreover, hypertension was shown to be associated with a profound downregulation of tissue hepatocyte growth factor, which is an essential endothelial growth factor. Thus, a paracrine effect induced by increased angiotensin II levels in hypertensive patients could potentially be involved in the regulation of EPC migration. Additionally, age and LDL cholesterol levels were shown to negatively correlate with EPC migration. This is in accordance with in vitro studies in differentiated endothelial cells, which demonstrate that aging and oxidized LDL can inhibit VEGF-induced endothelial cell migration (S.D., unpublished data, 2001). Thereby, oxidized LDL blocked VEGF-induced Akt activation and NO production, which are essential for endothelial cell migration. Interestingly, although the total number of risk factors for CAD was the best predictor for both reduced levels and the migratory capacity of EPCs, individual risk factors seem to differentially affect the number and the migratory capacity of EPCs. These data suggest that different mechanisms contribute to the impairment in EPC migration compared with the reduced levels of circulating EPCs.

Taken together, the present study demonstrates that EPC numbers and migratory capacity are impaired in patients with CAD, and this impairment relates to the number of risk factors for CAD. The direct effect of risk factors in persons without apparent CAD requires further investigations. However, given the important role of EPCs for neovascularization of ischemic tissue, these data not only give potential insight into the pathophysiological mechanisms contributing to impaired blood vessel formation in patients with CAD but may further provide the basis to more closely define the molecular

### TABLE 2. Multivariate Analysis

<table>
<thead>
<tr>
<th>EPC No.</th>
<th>CD34/KDR-Positive Cells</th>
<th>EPC Migration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>-0.187 0.178</td>
<td>-0.266 0.064</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td>0.110 0.375</td>
<td>-0.109 0.412</td>
</tr>
<tr>
<td><strong>Hypertension</strong></td>
<td>-0.177 0.174</td>
<td>-0.067 0.607</td>
</tr>
<tr>
<td><strong>Smoking</strong></td>
<td>-0.530 0.000</td>
<td>-0.317 0.018</td>
</tr>
<tr>
<td><strong>Family history of CAD</strong></td>
<td>0.030 0.803</td>
<td>-0.249 0.051</td>
</tr>
<tr>
<td><strong>LDL cholesterol</strong></td>
<td>0.007 0.955</td>
<td>-0.217 0.076</td>
</tr>
<tr>
<td><strong>Adjusted R²</strong></td>
<td>0.225 ...</td>
<td>0.373 ...</td>
</tr>
<tr>
<td><strong>Significance (ANOVA)</strong></td>
<td>... 0.003</td>
<td>... 0.000</td>
</tr>
</tbody>
</table>
pathways, which determine the fate of EPCs in patients at risk for CAD, to identify suitable therapeutic targets.

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