Reduced Number of Circulating Endothelial Progenitor Cells Predicts Future Cardiovascular Events

Proof of Concept for the Clinical Importance of Endogenous Vascular Repair

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Background—The maintenance of endothelial integrity plays a critical role in preventing atherosclerotic disease progression. Endothelial progenitor cells (EPCs) were experimentally shown to incorporate into sites of neovascularization and home to sites of endothelial denudation. Circulating EPCs may thus provide an endogenous repair mechanism to counteract ongoing risk factor–induced endothelial injury and to replace dysfunctional endothelium.

Methods and Results—In 120 individuals (43 control subjects, 44 patients with stable coronary artery disease, and 33 patients with acute coronary syndromes), circulating EPCs were defined by the surface markers CD34 and analyzed by flow cytometry. Cardiovascular events (cardiovascular death, unstable angina, myocardial infarction, PTCA, CABG, or ischemic stroke) served as outcome variables over a median follow-up period of 10 months. Patients suffering from cardiovascular events had significantly lower numbers of EPCs (P<0.05). Reduced numbers of EPCs were associated with a significantly higher incidence of cardiovascular events by Kaplan-Meier analysis (P=0.0009).

By multivariate analysis, reduced EPC levels were a significant, independent predictor of poor prognosis, even after adjustment for traditional cardiovascular risk factors and disease activity (hazard ratio, 3.9; P<0.05).

Conclusions—Reduced levels of circulating EPCs independently predict atherosclerotic disease progression, thus supporting an important role for endogenous vascular repair to modulate the clinical course of coronary artery disease. (Circulation. 2005;111:2981-2987.)

Key Words: atherosclerosis ■ coronary disease ■ stem cells, endothelial ■ endothelium ■ prognosis

The integrity and functional activity of the endothelial monolayer play a critical role in atherogenesis. Cardiovascular risk factors induce endothelial injury and a cascade of proinflammatory events, resulting in infiltration of monocytc cells and smooth muscle cell proliferation, which lead to the formation of atherosclerotic lesions. Ultimately, atherosclerotic plaque erosion and rupture cause myocardial infarction and sudden cardiovascular death. Therefore, the maintenance of endothelial integrity is of crucial importance for preventing the triggering of these processes.

Recent studies have identified a population of presumably bone marrow–derived cells, called circulating endothelial progenitor cells (EPCs), that can be isolated from bone marrow or circulating, blood-derived, mononuclear cells; express a variety of endothelial surface markers; incorporate into sites of neovascularization; and home to sites of endothelial denudation. Initial clinical studies demonstrated that risk factors for atherosclerosis are associated with reduced levels of circulating EPCs and that the functional integrity of the endothelium correlates with the activities of EPCs. These observations prompted the hypothesis that circulating EPCs may provide an endogenous repair mechanism to counteract ongoing risk factor–induced endothelial cell injury and to replace dysfunctional endothelium.

Therefore, we investigated whether levels of circulating EPCs correlate with atherosclerotic disease progression to establish a clinically meaningful role of ongoing endogenous endothelial repair mediated by circulating EPCs.

Methods

Patients and Control Subjects

The study population comprised 120 of 340 subjects randomly recruited in a single center between October 2000 and June 2004. Forty-four patients had stable coronary artery disease classified angiographically documented CAD and the absence of acute coronary syndromes (ACS) for 3 months before blood samples were analyzed.

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drawn. Thirty-three patients were studied with unstable CAD defined as de novo angina, crescendo angina, or angina at rest. Patients with ACS were further stratified for troponin T positivity to account for potential effects of myocardial necrosis on EPC levels. Extent of disease was quantified in all patients by the number of coronary arteries affected. Inclusion criteria were age from 18 to 85 years, male sex, hypertension, diabetes, smoking, and positive family history of cardiovascular disease (both non–ST-segment and ST-segment myocardial infarction). Exclusion criteria were clinical or biochemical evidence for the presence of concomitant inflammatory disease, chronic renal insufficiency (serum creatinine ≥1.4 mmol/L), impaired left ventricular ejection fraction (<45%), autoimmune or malignant disease, thrombocytopenia (<100 000/L), anemia (hemoglobin <8.5 g/dL), inability to understand the consent form, participation in or consent to participate in another study, previous coronary bypass surgery, severe peripheral arterial occlusive disease, or atrial fibrillation. Forty-three healthy subjects without any evidence of CAD by history and physical examination served as a control group. All study participants gave written informed consent, and the study was approved by the ethics committee of J.W. Goethe University (Frankfurt, Germany).

### Definition of Risk Factors for CAD

Hypertension was defined as a history of hypertension for >1 year requiring the initiation of antihypertensive therapy by the primary physician. Smoking was defined as a history of smoking >2 pack-years and/or smoking in the last year. Positive family history of cardiovascular disease was defined as evidence of premature CAD in a close relative (men <55 and women <65 years of age). Diabetic mellitus was defined as the need for oral antidiabetic drug therapy or insulin use. We calculated a score giving the risk of an individual subject to develop cardiovascular disease by considering age >65 years, male sex, hypertension, diabetes, smoking, and positive family history of CAD as single cardiovascular risk factors.

### Flow Cytometry

Detection of EPCs was performed as previously described. In brief, 100 μL peripheral blood was immunostained with monoclonal antibodies against human CD34 (Becton Dickinson; PerCP conjugated) and against human KDR (Sigma), followed by an PE-conjugated secondary antibody. Isotype-identical antibodies served as controls (Becton Dickinson). After incubation, cells were lysed, washed with PBS, and fixed in 4% paraformaldehyde before analysis of 70 000 events after exclusion of debris and platelets.

To assess the reproducibility of EPC measurements, circulating EPCs were measured twice from the same subjects (n=22) from 2 separate blood samples, revealing a very close correlation (r=0.86, P<0.0001).

### Long-Term Follow-Up

Clinical long-term follow-up was performed through a questionnaire sent to patients and telephone contact. All information about potential cardiovascular events was validated by source data, including analysis of coronary angiograms, discharge letters, or charts of hospital stays. Cardiovascular death was defined as death from myocardial infarction or documented sudden death. Unstable angina pectoris was defined as hospitalization for unstable angina pectoris of Braunwald IIIB or IIIB. Myocardial infarction was defined as an elevation of creatine kinase serum levels >2 times the upper limit of normal or new ST elevation (>0.1 mV) in ≥2 leads. Progression of coronary atherosclerosis was defined as the need for coronary revascularization of de novo lesions (percutaneous coronary intervention or bypass surgery) because of documented ischemia.

### Statistical Analysis

Data are expressed as mean±SD. Continuous variables were tested for normal distribution with the Kolmogorov-Smirnov test. Not normally distributed continuous variables (age, risk factor score, EPC numbers, extent of disease and high-sensitivity C-reactive protein [hs-CRP]) were compared by the Mann-Whitney U test. Comparisons between groups were analyzed by t test (2 sided) or ANOVA for normally distributed variables with >2 subgroups and by the Kruskal-Wallis test for nonnormally distributed variables. Post hoc range tests and pairwise multiple comparisons were performed with the t test (2 sided) with least-significant-difference adjustment. Comparison of categorical variables was generated by the Pearson χ² test. Multivariate linear regression analysis and nonparametric bivariate correlation (Spearman’s rank correlation coefficient) were used to correlate circulating EPC counts with

<table>
<thead>
<tr>
<th>TABLE 1. Patients’ Baseline Characteristics</th>
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<tr>
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<tr>
<td>---</td>
</tr>
<tr>
<td>Age, y</td>
</tr>
<tr>
<td>Male gender, n (%)</td>
</tr>
<tr>
<td>CVRF score, n (%)</td>
</tr>
<tr>
<td>Hypertension</td>
</tr>
<tr>
<td>Smoking</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>Family history</td>
</tr>
<tr>
<td>Troponin T positive</td>
</tr>
<tr>
<td>hs-CRP</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
</tr>
<tr>
<td>Extent of disease (1-/2-/3-vessel disease)</td>
</tr>
<tr>
<td>Statin therapy, n (%)</td>
</tr>
<tr>
<td>Aspirin/clopidogrel, n (%)</td>
</tr>
<tr>
<td>ACE inhibitor/AT1 blocker, n (%)</td>
</tr>
</tbody>
</table>

CVRF indicates cardiovascular risk factor score; Co, control subjects. Baseline characteristics for patients with ACS vs troponin-positive or -negative values are identical.
cardiovascular risk factors. To identify independent determinants of EPC numbers, a multivariate linear regression analysis for various cardiovascular risk factors was performed. Cumulative event-free survival was univariately evaluated by Kaplan-Meier analysis (log-rank test). Cox proportional-hazard ratio was used to estimate the relative risk for major adverse cardiac events and the association with identified variables. Hazard ratios (HR) and 95% CIs are given. We considered sensitivity and specificity for the identification of high-risk patients of equal importance. Therefore, in receiver-operating characteristic (ROC) curve analyses, the best prognosticator for event-free survival was considered to be the parameter that gave the highest product of sensitivity and specificity for predicting major adverse cardiac events. Statistical significance was assumed if a null hypothesis could be rejected at $P \leq 0.05$. All statistical analysis was performed with SPSS, version 11.5 (SPSS Inc).

**Results**

The baseline characteristics of the 120 subjects are summarized in Table 1. As expected, patients with documented CAD had a significantly higher number of risk factors, were slightly but significantly older, and more frequently were treated with statins, platelet inhibitors, and ACE inhibitors/AT-1 receptor blockers. Patients with ACS differed from patients with stable CAD with respect to the incidence of smoking, hs-CRP serum levels, and LDL serum cholesterol levels, which were all higher in patients with ACS. In addition, patients with ACS were less likely to be treated with statins before inclusion in the study.

### Determinants of Circulating EPC Levels

Figure 1A shows that control subjects had significantly higher levels of EPCs compared with patients with documented CAD. By univariate analysis for the entire cohort, the classic risk factors of age, hypertension, smoking, and family history of CAD, as well as disease activity and extent of coronary atherosclerotic involvement, were inversely correlated with the number of circulating EPCs (Table 2). Figure 1B and 1C illustrates the inverse relation between age and

**Table 2. Univariate Correlation Between Disease Activity, Cardiovascular Risk Factors, and CD34$^{+}$KDR$^{-}$EPCs in 120 Individuals**

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>$R$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.220</td>
<td>0.008</td>
</tr>
<tr>
<td>Hypertension</td>
<td>-0.297</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Smoking</td>
<td>-0.178</td>
<td>0.026</td>
</tr>
<tr>
<td>Family history</td>
<td>-0.252</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Diabetes</td>
<td>-0.114</td>
<td>0.104</td>
</tr>
<tr>
<td>Disease activity</td>
<td>-0.390</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CVRF score</td>
<td>-0.376</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Extent of disease</td>
<td>-0.320</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>-0.080</td>
<td>0.455</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>0.144</td>
<td>0.059</td>
</tr>
</tbody>
</table>

CVRF indicates cardiovascular risk factor score.
EPC levels and the reduced EPC levels in subjects with a positive family history of premature CAD, respectively. By multivariate analysis, age and a positive family history of CAD remained the only significant independent predictors of a reduced number of EPCs (Table 3).

Although we did not detect a significant augmentation of EPCs in patients with ACS, which likely reflects the early blood sampling, we repeated both the univariate and multivariate analyses in the subcohort restricted to control subjects and stable CAD patients (n=87). As illustrated in Table I of the Data Supplement, when patients with ACS were excluded, identical results were obtained with age and family history as the only independent significant predictors for reduced EPC levels. Finally, even with the analysis restricted to the subgroup of healthy control subjects only (n=43), age and a positive family history of premature CAD independently predicted reduced EPC levels (Table II in the Data Supplement), confirming the strong influence of these risk factors on EPC levels.

**Circulating EPC levels and Atherosclerotic Disease Progression**

The median duration of follow-up was 10.0±12.1 months (range, 1 to 48 months). During follow-up, a total of 11 patients experienced a cardiovascular event (Table 4).

![Image](314x464 to 548x726)

Table 2. ROC curve showing correlation between major adverse cardiac events and levels of circulating CD34/KDR^+ -EPCs. R^2 = 0.769. R^2 = 1.0 denotes perfect correlation; R^2 = 0.5 denotes no correlation.

Patients suffering from a cardiovascular event during follow-up had significantly lower EPC levels at inclusion in the study, with 0.0067±0.0097 per 100 peripheral mononuclear cells (PMNCs) compared with 0.02±0.02 per 100 PMNCs (P<0.05) in patients without a cardiovascular event.

When patients were categorized into quintiles according to EPC levels, those patients in the lowest quintile had a significantly (P<0.05) higher incidence of cardiovascular events during follow-up. To maximize the predictive power of EPC levels, we used ROC curve analysis over the entire dynamic range of EPC numbers to identify the threshold level for EPCs providing the highest predictive value for the occurrence of cardiovascular events during follow-up (Figure 2). Kaplan-Meier analysis revealed a significantly increased incidence of cardiovascular events in those patients with levels of circulating EPCs below the threshold (CD34^+KDR^-EPC ≤0.0038) identified by ROC curve analysis for maximized predictive value (Figure 3). Table 5 illustrates that the crude HR, measured by the Cox proportional-hazard regression model, for suffering a cardiovascular event during follow-up was 6.3 (P=0.003). As expected, the presence of ACS had an impact on prognosis (univariate HR, 2.03; 95% CI, 1.2 to 3.5; P=0.006).

However, even when adjusted for disease activity and overall risk factor load for CAD, low numbers of circulating EPCs were associated with a significantly, ~4-fold, increased risk of suffering a cardiovascular event during follow-up (Table 5). Thus, the number of circulating EPCs independently predicts atherosclerotic disease progression.

**Discussion**

The results of the present study confirm and extend previous reports suggesting that the levels of circulating EPCs may be a surrogate marker of vascular function and cumulative cardiovascular risk. Most importantly, however, to the best
of our knowledge, this is the first study to document that reduced levels of circulating EPCs independently predict atherosclerotic disease progression. Thus, our data provide clinical evidence for the hypothesis that circulating EPCs contribute to ongoing vascular repair.

Endothelial cell injury is regarded as the classic stimulus for the development of atherosclerotic lesions. Indeed, not only do classic risk factors for atherosclerosis induce endothelial injury, but impaired endothelial function predicts the risk of subsequent cardiovascular events. Ultimately, endothelial damage represents a balance between the magnitude of injury and the capacity for repair. Recent experimental studies suggest that EPCs may contribute to ongoing endothelial repair by providing a circulating pool of cells that can home to denuded parts of the artery after balloon injury or could replace dysfunctional endothelial cells.

An impairment of this repair capacity may affect atherosclerotic disease progression. We have previously shown that classic risk factors for atherosclerosis are associated with reduced number and function of circulating EPCs. More recently, levels of circulating EPCs were shown to correlate with endothelial vasodilator function, and coronary collateral support in patients with CAD, suggesting an important role of circulating EPCs in vascular homeostasis. The present study now documents that the level of circulating EPCs is an independent predictor of future cardiovascular events. Thus, taken together, these findings suggest that circulating EPCs are important for maintaining the functional integrity of the endothelial monolayer and exert important functions of vascular repair of continuous risk factor–induced endothelial injury.

The reduction in circulating EPC number may be secondary to a variety of mechanisms: exhaustion of the pool of progenitor cells in the bone marrow, reduced mobilization, or reduced survival and/or differentiation. The significant inverse correlation between patient age and levels of circulating EPCs reported previously and in the present study may indicate that continuous endothelial damage will lead to an eventual depletion or exhaustion of a presumed finite supply of EPCs. In analogy to the lympho-hematopoietic stem cell system in which basal hematopoiesis is maintained in aging but the capacity to react to stress-induced mobilization gradually declines with increased age, atherosclerosis-prone apolipoprotein E mice exhibit significantly reduced vascular progenitor cells in the bone marrow with increased age. Moreover, risk factors for atherosclerosis most likely directly influence the mobilization and survival of EPCs via impairing nitric oxide bioavailability. Indeed, mice deficient in endothelial nitric oxide synthase demonstrate a profound impairment in ischemia- or exercise-induced mobilization of EPCs. In addition, EPCs derived from high-risk patients become senescent more rapidly, a process in part reversible by stimulating the Akt–nitric oxide synthase pathway. Finally, risk factors may modulate the mechanisms that facilitate homing and differentiation of circulating EPCs. Thus, it is most likely that risk factors for atherosclerosis synergistically act on a variety of mechanisms that culminate in reduced levels of circulating EPCs. However, the lack of

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**Figure 3.** Event-free survival according to levels of circulating CD34^+^ KDR^+^-EPCs defined by ROC curve analysis.

**TABLE 5. Crude, Disease Activity–Adjusted, and Risk Factor–Adjusted Relative Risks of a First Major Cardiovascular Event in Patients With Low EPC Counts**

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR for MACE (95% CI)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Crude relative risk</td>
<td>6.3 (1.8–21.8)</td>
<td>0.003</td>
</tr>
<tr>
<td>Disease activity–adjusted relative risk</td>
<td>4.2 (1.1–16.0)</td>
<td>0.032</td>
</tr>
<tr>
<td>Risk factor– and disease activity–adjusted relative risk</td>
<td>3.9 (1.1–14.6)</td>
<td>0.036</td>
</tr>
</tbody>
</table>

MACE indicates major adverse cardiovascular event.
correlation between CRP and circulating EPC levels may indeed indicate that levels of circulating EPCs not only are a marker of an unspecified inflammation but also reflect endogenous vascular repair capacity in the presence of ongoing risk factor–induced endothelial injury. Clearly, the nature of our clinical study does not permit us to dissect the individual components leading to the impaired vascular repair capacity associated with reduced levels of EPCs. In addition, impaired functional activity of the EPCs may further amplify the reduced repair capacity. Indeed, in a subset of our patients, the number of CD34+/KDR⁺–EPCs detected by fluorescence-activated cell sorter analysis correlated closely with the migratory capacity to vascular endothelial growth factor (r = 0.47, P < 0.01; n = 31) and with an EPC culture assay (r = 0.46, P < 0.02; n = 26). Thus, further studies should investigate whether functional properties of EPCs such as migratory capacity or colony forming capacity may provide additional prognostic information in addition to simply measuring the number of circulating EPCs. Likewise, assessing the number of the more immature circulating EPCs, as defined by the surface marker CD133, may provide additional mechanistic insights.

In summary, reduced levels of circulating EPCs independently predict future cardiovascular events, thus supporting an important role for endogenous vascular repair to modulate the clinical course of CAD. Importantly, statin therapy and physical exercise, both of which are known to exert beneficial effects in primary and secondary prevention of atherosclerosis, were recently shown to augment the number and function of EPCs. Thus, monitoring the levels of circulating EPCs as a surrogate biological marker might be specifically useful for identifying novel therapeutic approaches targeted to enhance endogenous vascular repair capacity and thereby modify the progression of cardiovascular disease.

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References


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