Increase of Circulating Endothelial Progenitor Cells in Patients with Coronary Artery Disease After Exercise-Induced Ischemia

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Objectives—The concept of neovascularization in response to tissue ischemia has been extended by the finding of postnatal vasculogenesis initiated by endothelial progenitor cells (EPCs). The aim of this study was to analyze whether a maximal stress test in patients with coronary artery disease (CAD) increases the number of circulating EPCs.

Methods and Results—Blood concentration of EPCs was analyzed by FACS and cell culture assay in CAD patients with (n=16) or without (n=12) exercise-induced myocardial ischemia and in healthy subjects (n=11) for up to 144 hours after maximal stress test. Plasma concentrations of vascular endothelial growth factor (VEGF), basic fibroblast growth factor, tumor necrosis factor-α, and granulocyte macrophage–colony stimulating factor were determined by ELISA. EPCs increased significantly in ischemic patients, with a maximum after 24 to 48 hours (cell culture: 3.3±0.5-fold increase; FACS: 3.1±0.6-fold increase) and returned to baseline within 72 hour. In nonischemic patients and healthy subjects, no EPC increase was detectable. VEGF levels in ischemic patients increased significantly after 2 to 6 hours (maximum after 2 hours: 4.0±1.1-fold increase) and no change was observed in nonischemic patients and healthy subjects; ΔVEGF and ΔEPC correlated significantly (r=0.66).

Conclusions—Patients with symptomatic CAD respond to a single episode of exercise-induced myocardial ischemia with a time-dependent increase in circulating EPCs. This increase may be related to and preceded by an increase in plasma VEGF. (Arterioscler Thromb Vasc Biol. 2004;24:684-690.)

Key Words: exercise ■ growth factor ■ cytokines ■ endothelium

Ischemia represents the most potent stimulus for postnatal vascular growth and remodeling. For a long time, it was believed that collateralization, eg, in-limb ischemia, occurs primarily by neovascularization, ie, sprouting of preexistent vessels (angioneogenesis). In recent years, this traditional model has been greatly extended by the finding that augmentation of neovascularization may also be promoted by bone marrow-derived endothelial progenitor cells (EPCs), which are capable to form entirely new vessels into the ischemic tissue, ie, by vasculogenesis.1-4

Based on this finding, the therapeutic potential of EPCs expanded ex vivo for myocardial and limb ischemia was first evaluated in animal models3,5 and recently applied in humans.6,7 It could be demonstrated that the intravenous injection of EPCs into LAD-ligated rats, for example, attenuated the pathologic left ventricular remodeling and improved myocardial blood flow.8 The myocardial neoangiogenesis resulted in decreased cardiomyocyte apoptosis in the peri-infarct region, reduction in collagen deposition, and sustained improvement of ventricular function.9 Increases in circulating cytokines, especially vascular endothelial growth factor (VEGF), have been shown to mobilize EPCs from bone marrow into the peripheral blood.3,10,11

Despite the recent advances in EPC research, the mechanisms stimulating the increase in circulating EPC numbers are still not fully understood. Prolonged irreversible ischemia (ie, in myocardial infarction) has been shown to trigger EPC release.12 It remains unclear, however, whether a single episode of reversible ischemia is sufficient to induce any measurable increase in EPC numbers. Aim of this prospective clinical study was to determine the time course of circulating EPC numbers after a single symptom-limited exercise stress test in patients with stable coronary artery disease (CAD).

Methods

Inclusion Criteria

Patients with angiographically documented stenotic CAD (>75%) and exercise-induced myocardial ischemia (IP), revascularized
asymptomatic patients with nontenent CAD (<50%) and no exercise-induced myocardial ischemia (control patients [CP]), and age-matched healthy subjects (HS) were included. All probands had a normal left ventricular function (LVEF) (>60%). Exclusion criteria were left main CAD (>50% lumen diameter reduction), significant valvular heart disease, and orthopedic or other conditions prohibiting participation in the ergometer stress test.

The protocol of this study was approved by the Ethics Committee of the University of Leipzig, and written informed consent was obtained from all patients and subjects at study begin.

Maximal Exercise Test
Maximal exercise testing was performed on a calibrated electronically braked bicycle in an upright position. ECG tracing and blood pressure readings were obtained every minute. Workload was increased progressively every 3 minutes in steps of 25 W, beginning at 25 W. Myocardial ischemia was confirmed by the presence of typical angina pectoris or a significant exercise-induced ST-segment depression (>0.1 mV descending or horizontal). Exercise was terminated when patients experienced progressive anginal chest pain, physical exhaustion, or when 3-mm horizontal ST-segment depression was reached. Duration of ischemia was defined as the time interval from beginning of angina pectoris/ST-segment depression to spontaneous resolution of symptoms.

Collection of Venous Blood Samples
Before and after (at hours 2, 4, 6, 8, 24, and 48) a single maximal symptom-limited exercise test on a bicycle ergometer, venous blood samples were taken. Cultivation of EPCs was performed from samples before and at 8 hours, 24 hours, and 48 hours after the maximal exercise test, whereas the FACS analysis was performed from all samples. From a subset of patients and controls (IP, n = 7; CP, n = 9; HS, n = 8), blood samples for FACS analysis and EPC culture were collected for an extended period of up to 144 hours after the stress test.

Isolation, Cultivation, and Characterization of EPCs
Mononuclear cells (MNC) were isolated by density gradient centrifugation from 20 mL of peripheral blood as described in the current literature. After isolation 2 × 10^6 cells were plated on gelatin-coated 4-well chamber slides (NUNC, Wiesbaden, Germany) in medium 199 containing 20% fetal calf serum (Gibco), endothelial cell growth supplement (CCPro, Neustadt, Germany), and antibiotics (100 U penicillin/100 μg/mL streptomycin; Gibco). After 4 days in culture, nonadherent cells were removed by a thorough rinsing with cell culture medium, and adherent cells were characterized by cytochemical methods. For long-term culture up to 4 weeks, the cells were kept under standard conditions and the medium was changed every third day.

To characterize the adherent cells, immunofluorescent staining with endothelial specific markers (von Willebrand Factor [vWF]; Dako, Hamburg Germany), CD-31 (ImmunoTech), or the leucocyte marker CD-45 (Caltac) was performed.

To investigate the phenotype of the attached cells by FACS analysis, the cells were harvested after 4 days in culture by laser scanning cytometry (LSC) (CompuCyte). To quantify the content of circulating EPCs by FACS analysis, a volume of 200 μL peripheral blood was incubated for 20 minutes with biotinylated anti-human KDR (R&D Systems) followed by the addition of allophycocyanin conjugated mouse anti-human CD34 (BD Pharmingen), PerCP conjugated mouse anti-human CD3, and FITC-labeled streptavin (Dako), and a further incubation in the dark for 20 minutes. After incubation, the erythrocytes were lysed, and the remaining cells were washed with PBS and fixed in 2% paraformaldehyde before analysis using a FACS Calibur (Becton-Dickinson).

To quantify the amount of KDR^CD34^ double-positive cells, the mononuclear cell fraction was gated and analyzed for the expression of CD34 and CD3. Only the CD34^-CD3^ cells were finally investigated for the content of KDR^CD34^ double-positive cells. Mixing experiments revealed that cells with a frequency as low as 0.005% could be detected by FACS analysis.

Plasma VEGF, Granulocyte Macrophage Colony-Stimulating Factor, Tumor Necrosis Factor-α, and Basic Fibroblast Growth Factor Levels
Plasma levels of cytokines were measured by high-sensitivity ELISA assays (R&D Systems, Wiesbaden, Germany) according to the manufacturers instructions. Results were compared with standard curves, and the lower detection limits were: VEGF, 9 pg/mL; granulocyte macrophage colony stimulating factor (GM-CSF), 0.4 pg/mL; tumor necrosis factor α (TNF-α), 0.18 pg/mL; basic fibroblast growth factor (b-FGF), 0.22 pg/mL. The intra-assay and interassay variability was <10%. Measurements were performed in duplicate.

Statistical Analysis
Data are expressed as mean ± SEM. Continuous variables were tested for normal distribution with the Kolmogorov-Smirnov test and compared among groups by 1-way ANOVA. Intragroup comparisons were performed using a nonparametric test (Wilcoxon test). A P < 0.05 was considered statistically significant.

Results
Patient Characteristics
In the present study, 16 patients with stenotic CAD and exercise-induced myocardial ischemia (IP), 12 CAD patients with nonischemic CAD (CP), and 11 healthy subjects (HS) were included. The 3 groups did not differ in age, body mass index (BMI), or left ventricular ejection fraction (Table). No difference with regard to the cardiovascular risk profile (hypertension, diabetes mellitus, smoking) and current medication was evident between the IP and CP groups. The severity of the disease, as documented by the CCS class, was different between the IP and CP groups (Table). As expected, CP and HS reached a significantly higher maximal workload during the exercise test. Presence of myocardial ischemia in the IP was confirmed by significant ST-segment depression (13/16) and/or typical angina pectoris (12/16) at peak exer-
Characterization of EPCs

When plated on gelatin-coated tissue culture dishes, attached cells adopted a spindle-shaped morphology after 4 days in culture (Figure 1A). Using laser scanning cytometry to evaluate the amount of attached cells per well, 1.5 × 10^6 ± 2500 cells were counted, resulting in a frequency of ~0.75% attached cells of originally plated MNCs. FACS analysis confirmed that the majority of attached cells express cell surface markers consistent with endothelial lineage cells (VE cadherin 73.0 ± 6.8%; KDR 58.2 ± 6.1%) (Figure 1A). Furthermore, the positive immuno-histochemical staining with anti-vWF (Figure 1B), anti-CD31 (Figure 1C), and the positive amplification of eNOS (Figure 1D lane 4) and vWF mRNA (Figure 1D lane 5) by RT-PCR confirmed the endothelial phenotype. Further characterization excluded significant contamination by adherent monocytes (negative staining for CD45; Figure 1C) and T cells (FACS analysis for CD3; Figure 1A).

To analyze the fate of the attached cells after 4 days in culture, the cells were cultured for up to 4 weeks. As documented by light microscopy, a cobble-stone morphology is adopted by the cells after 4 weeks in culture (Figure 2), a phenomenon characteristic for endothelial cells. The differentiation toward endothelial phenotype is further confirmed by the increasing expression of vWF (Figure 2).

Effects of Reversible Myocardial Ischemia on Circulating Endothelial Progenitor Cells

Two independent methods, a cell culture assay and FACS analysis, were used to assess the effect of a short ischemic period on the number of circulating EPCs. In the cell culture assay, the EPCs were determined as attached cells double-stained for FITC-UEA-1 and Di-LDL^13 (Figure 1A and B, available online at http://atvb.ahajournals.org). To evaluate the number of double-positive stained cells, an LSC-based method was applied (Figure IC through IE, available online at http://atvb.ahajournals.org).

Before starting the maximal symptom-limited exercise test, the total number of EPCs as determined by the cell culture assay was somewhat lower but not significantly reduced in the group of CAD patients with exercise-induced myocardial ischemia (IP) as compared with the CAD patients without ischemia (CP) or the healthy controls (HS) (IP, 1250 ± 255; CP, 1623 ± 516; HS, 1719 ± 547; P = 0.71). The number of double-positive stained EPCs in the IP group was observed as compared with the begin or to the CP/HS group (24 hours after exercise test: IP 2.9 ± 0.4-fold increase, P = 0.001 versus pre-exercise and P = 0.02 versus CP and HS; 48 hours after exercise test: IP 3.3 ± 0.5-fold increase, P = 0.001 versus pre-exercise and P = 0.01 versus CP and HS). In the IP group, EPC levels returned to normal within 72 hours (Figure 3). No significant difference was detected in IP subgroup analysis with regard to EPC release in response to reversible myocardial ischemia and cardiovascular risk factors like smoking, diabetes mellitus, hypertension, or hyperlipidemia.

Before starting the symptom-limited exercise test, the amount of CD34^+ /KDR^+ cells/mL as quantified by FACS analysis was somewhat lower in the IP patients as compared with the CP or the HS groups but did not reach statistical significance (IP: 73.9 ± 7.6 cells/mL blood; CP: 77.4 ± 13.1 cells/mL blood; HS: 97.6 ± 18.7 cells/mL blood; P = 0.45). After the single exercise test, a significant increase in Di-LDL/UEA-1 double-positive stained EPCs in the IP group was observed as compared with the begin or to the CP/HS group (24 hours after exercise test: IP 2.9 ± 0.4-fold increase, P = 0.001 versus pre-exercise and P = 0.02 versus CP and HS; 48 hours after exercise test: IP 3.3 ± 0.5-fold increase, P = 0.001 versus pre-exercise and P = 0.01 versus CP and HS). In the IP group, EPC levels returned to normal within 72 hours (Figure 3). No significant difference was detected in IP subgroup analysis with regard to EPC release in response to reversible myocardial ischemia and cardiovascular risk factors like smoking, diabetes mellitus, hypertension, or hyperlipidemia.
Plasma Concentration of VEGF, GM-CSF, b-FGF, and TNF-α

In IP patients, a single ischemic exercise period led to a significant time-dependent increase of VEGF in the plasma after 2 to 6 hours of the maximal exercise test, with a maximum after 2 hours (4.0 \pm 1.1-fold increase, \( P=0.001 \) versus pre-exercise, \( P=0.03 \) versus CP, HS; beginning: 14.0 \pm 4.1 pg/mL versus 2 hours: 57.3 \pm 17.2 pg/mL; \( P<0.05 \)). No increase was detected in the CP (beginning: 15.4 \pm 2.5 pg/mL versus 2 hours: 21.7 \pm 3.0 pg/mL; \( P=\text{NS} \)) or the HS group (beginning: 14.8 \pm 3.0 pg/mL versus 2 hours: 15.8 \pm 3.0 pg/mL; \( P=\text{NS} \)). For GM-CSF, TNF-α, or b-FGF no significant changes were observed in all 3 groups. A linear correlation was found between the maximal increase in VEGF plasma concentration and the maximal increase of Di-LDL/lectin double-positive stained cells (\( r=0.66, P<0.0001; \) Figure II, available online at http://atvb.ahajournals.org).

Discussion

In patients with exercise-induced myocardial ischemia, a single symptom-limited exercise test is able to: (1) increase the number of circulating endothelial precursor cells, with a maximum after 24 to 48 hours and (2) increase the serum concentration of VEGF after 2 to 6 hours while leaving the plasma levels of GM-CSF, TNF-α, or b-FGF unchanged.

These observations imply that a single episode of reversible exercise-induced myocardial ischemia is sufficient to increase the plasma level of VEGF and the amount of circulating endothelial progenitor cells. This finding is consistent with the hypothesis that an ischemic stimulus may trigger the release of EPCs from the bone marrow in the peripheral blood.

Characterization of EPCs

A central role for the significance of the findings reported here is the careful characterization of the investigated cell population. In accordance with reports in the literature,5,12,16 the analysis of the MNC cell fraction, which attached to gelatin-coated plates in endothelium-specific medium, revealed that these cells were double-positive for acetylated (Ac)-LDL uptake and lectin binding. An estimation from laser scanning cytometry data revealed that \( \approx 0.2\% \) of originally isolated MNCs can be regarded as EPCs, in accordance with other published data.13 To exclude that these cells are attached monocytes or macrophages, which are also capable for LDL uptake and lectin binding,17,18 additional characterization is necessary. As demonstrated by FACS analysis and

Figure 1. Characterization of attached MNCs by FACS analysis (A), immunofluorescent staining (B, C), and RT-PCR (D).

Figure 2. Freshly isolated MNCs were grown in endothelium-specific media, and microscopic pictures were taken after 4 days, 10 days, and 4 weeks. After 4 weeks in culture, the morphology changed and cobble-stone cells were visible. This change in morphology toward endothelial cells is accompanied with a significant increase of vWF expression in the cells as detected by RT-PCR. Lane 1 indicates negative control (no template added to the PCR reaction); lane 2, after 4 days; lane 3, after 4 weeks; and lane 4, HUVEC cells as positive control. For quantitative analysis, the ratio of vWF/GAPDH is depicted.

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RT-PCR, the attached cells, which are analyzed and quantified in this study, exhibit an endothelial phenotype, positive staining for CD31, vWF, VE-cadherin, and expression of eNOS. They are not monocytes or adherent macrophages, as demonstrated by the negative staining of CD3 and CD45. The endothelial phenotype is further supported by the observation that in long-term culture the cells adopted a cobble-stone morphology, typical for endothelial cells, and express vWF in the same magnitude as differentiated endothelial cells (HUVECs).

Increase in Circulating EPCs in Response to Ischemia

The development of new vessels in response to tissue ischemia constitutes a natural reaction intended to maintain adequate perfusion. However, this process was believed to result from the sprouting of preexistent arteries rather than from the formation of entirely new vessels. This traditional concept was shattered by the finding that operative hind limb ischemia induces a significant increase of circulating bone marrow-derived EPCs and that heterologous, homologous, or autologous transplanted EPCs are incorporated into foci of neovascularization. These results from animal models have also been confirmed in the clinical setting. In patients with acute myocardial infarction, an increase of EPCs and a possible participation in neovascularization have been demonstrated.

Despite these advances in EPC research, many questions remain to be answered. Is a single episode of reversible myocardial ischemia sufficient to have any effect on the number of circulating EPCs? The present study is the first to report that a single episode of myocardial ischemia is sufficient to trigger a time-dependent increase of circulating EPCs. The observation that the number of EPCs increased only in patients with exercise-induced myocardial ischemia and not in the CAD patients without an induced ischemia or the healthy controls confirms the role of the ischemic stimulus as the initiating factor for EPC increase. A single ischemic stimulus led to a 3.3-fold increase of circulating EPCs. This augmentation in EPCs is comparable with the response to other therapeutic or pathophysiological stimuli resulting in an increase of EPCs, like statin treatment for 4 weeks, myocardial infarction, or VEGF gene therapy. The transitory increase of circulating EPCs after a single episode of reversible myocardial ischemia reached a maximum after 24 to 48 hours. This finding significantly extends our knowledge about the time course of changes in EPC levels. Two recent reports support the concept of early changes in EPC numbers in response to external stimuli. In a cell culture model, Dimmel et al observed a maximum of differentiated adherent EPCs after 48 hours of incubating isolated MNC with a HMG-CoA reductase inhibitor (atorvastatin). In the clinical context, Gill et al reported a significant transient increase of EPCs 6 to 12 hours after a vascular trauma (coronary artery bypass surgery or burn injury). So far, only cross-sectional data have been published with regard to ischemia-related EPC release in CAD. Shintani et al observed higher levels of circulating EPCs 7 days after an acute myocardial infarction as compared with a control group of patients without evidence of CAD. Although the net
increase in circulating EPCs was similar to our finding, the studies differ as far as the time course is concerned. It may well be that in the setting of complete vascular occlusion, the late release of VEGF and the associated increase of EPCs may be related to the revascularization procedure rather than to the acute myocardial infarction

Patients with exercise-induced ischemia showed a somewhat reduced amount of circulating EPCs at baseline. Although the difference in cell number did not reach statistical significance, these data are in accordance with recent observations that the severity of the disease or number of risk factors influence the amount of circulating progenitor cells.22,23

Factors Responsible for the Release of EPCs
What is the potential trigger for increasing circulating EPC numbers after an exercise-induced myocardial ischemia? Inflammatory cytokines and tissue growth factors are released from the tissue in response to an ischemic injury. Gene targeting studies have demonstrated that VEGF not only is essential for vascular sprouting (angiogenesis) but also is required for vasculogenesis.24,25 The role of VEGF for stimulating the release of EPCs has been confirmed in animal and human studies: The gene transfer of VEGF into the ischemic myocardium26 or ischemic hind limb11,26 enhances the population of circulating EPCs by a factor of 2 to 4 over baseline.

In the present study, we observed a significant increase in VEGF plasma concentration after 2 to 6 hours, with a maximum 2 hours after the initial ischemic trigger. This observation is in accordance with animal studies demonstrating a significant increase of plasma VEGF even 5 minutes after LAD ligation.27 The elevation was maintained for up to 2 hours, the last time point measured in this study. Based on the observation that gene transcription/translation takes at least 6 to 8 hours, one has to assume that this rapid increase in plasma VEGF is primarily caused by stimuli-mediated release of VEGF from internal stores or secreted by monocytes,28 mast cells,29 macrophages,30 or erythroid cells.31 One important factor regulating VEGF secretion may be protein kinase B, also known as Akt. In a cell culture model, it could be documented that the transfection with a permanent activated Akt leads to HIF-1–independent increase in VEGF secretion.32 Nevertheless, further extensive studies are necessary to determined which cells are involved in the rapid VEGF release and to elucidate the exact signal cascade between the ischemic tissue and the increase in the VEGF levels.

The concept that VEGF is an important humoral factor for EPC mobilization/differentiation is supported by the correlation between the increase in VEGF serum concentration and the increase of circulating EPCs previously described after intramuscular VEGF gene therapy.26 Moreover, the administration of VEGF into mice (administered daily over a period of 7 days) resulted in a significant increase of circulating EPCs 24 hours after the initial VEGF injection was administered.11 This time course from the first VEGF application to the significant increase in circulating EPCs in these animal experiments is similar to the results that we are reporting in the present study, in which the significant EPC increase follows the VEGF release after 24 hours. Because the plasma concentration of VEGF in ischemic situations are comparable in studies investigating patients with myocardial infarction or peripheral arterial occlusive disease,12,33 these observations imply that the increase in plasma VEGF is independent from the ischemic tissue mass and that VEGF may be released from other tissues by humoral factors.

In contrast, the serum concentration of other investigated cytokines, ie, b-FGF, GM-CSF, and TNF-α, did not change after the ischemic episode. Again, these findings are in accordance with previously published results.12,13,22

Despite the intriguing correlation between peak VEGF and peak EPC levels, one should keep in mind that, for example, statins use a VEGF-independent pathways to increase the number of circulating EPCs.20 Nevertheless, both induction pathways seem to merge at the level of PI3K/Akt activation. As recently shown, the pharmacological inhibition of PI3K20 or the overexpression of a dominant negative Akt construct20,34 abolished the statin and the VEGF-induced hematopoietic progenitor cell differentiation.

The results of this study may have important clinical implications. Exercise above the ischemic threshold is associated with increased risk of coronary events and ventricular arrhythmias in stenotic CAD, whereas it is safe and feasible in patients with peripheral vascular disease (PVD). One might hypothesis that exercise training over a longer time period, a well-established therapeutic concept in PVD patients, may lead to a solid increase in circulating EPCs, thereby enhancing the neovascularization and alleviation of symptoms. Nevertheless, based on investigations showing that the quality and quantity of mobilized EPCs are impaired,22,23 it is tempting to assume that the increase of circulating EPCs by a single ischemic bout from the bone marrow may not be enough to correct cardiac function or contractility. Therefore, the local delivery of a high concentration of ex vivo cultivated EPCs may be necessary to elicit a therapeutic benefit, as recently demonstrated in clinical studies.7,35

In conclusion, the present study demonstrates for the first time to our knowledge that a short episode of myocardial ischemia in CAD patients is sufficient to induce a considerable increase in the number of circulating EPCs, most likely resulting from enhanced differentiation of EPCs from circulating pluripotent bone marrow-derived progenitor cells. This increase seems to be related to the increase of plasma VEGF.

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References


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Figure I

A

B

C

D

E

FITC-lectin vs. DI-LDL scatter plots showing fluorescence intensity distribution.
Figure II

$\Delta$ EPC content vs $\Delta$ VEGF

$r = 70$
$p < 0.0001$
Figure Legends

**Figure I:**
Representative fluorescence photomicrograph and LSC scatter diagrams are shown. EPCs after 4 day cell culture of a CAD patients before (A) and after 48 h (B) of a symptom-limited exercise test. EPCs were identified as Di-LDL and FITC-conjugated UEA-1 double positive stained cells (orange stained cells). To set the quadrant for the LSC evaluation cells only stained with FITC-UEA-1 (C) or Di-LDL (D) are measured. As soon as the cells were stained with both dyes a shift of cells into the upper right quadrant is visible (E).

**Figure II:**
The correlation between changes in the VEGF plasma concentration and the change in the amount of EPC in the circulation is depicted.