Effects of Exercise and Ischemia on Mobilization and Functional Activation of Blood-Derived Progenitor Cells in Patients With Ischemic Syndromes

Results of 3 Randomized Studies

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Background—Exercise training (ET) has been shown to improve regional perfusion in ischemic syndromes. This might be partially related to a regeneration of diseased endothelium by circulating progenitor cells (CPCs) or CPC-derived vasculogenesis. The aim of the present study was to determine whether ischemic stimuli during ET are required to promote CPC mobilization in patients with cardiovascular diseases.

Methods and Results—Patients with peripheral arterial occlusive disease (PAOD) were randomized to 4 weeks of daily ischemic ET or control (group A). Successfully revascularized patients with PAOD were randomized to 4 weeks of daily nonischemic ET or control (group B). Patients with stable coronary artery disease were subjected to 4 weeks of subischemic ET or control (group C). At baseline and after 4 weeks, the number of KDR/CD34/CPCs was determined by fluorescence-activated cell sorting analysis. Levels of vascular endothelial growth factor (VEGF) were measured by ELISA. A Matrigel assay was used to quantify CPC integration into vascular structures. Expression of the homing factor CXCR4 was determined by reverse transcription–polymerase chain reaction. In group A only, ischemic ET increased VEGF levels by 310% (P<0.05 versus control) associated with an increase in CPCs by 440% (P<0.05 versus control), increased CXCR4 expression, and enhanced integration of CPCs into endothelial networks. In contrast, subischemic ET in groups B and C increased CXCR4 expression and CPC integration.

Conclusions—In training programs, symptomatic tissue ischemia seems to be a prerequisite for CPC mobilization. However, ischemic and subischemic ET programs affect CXCR4 expression of CPCs, which might lead to an improved CPC integration into endothelial networks. (Circulation. 2005;111:3391-3399.)

Key Words: angiogenesis ■ exercise ■ stem cells

In stable ischemic vascular syndromes, exercise has been empirically used to improve symptom-free exercise capacity and regional perfusion in ischemic tissues. Several important pathways by which exercise training (ET) may enhance perfusion were identified in recent years: (1) shear stress–associated improvement of endothelial function, with increased endothelial nitric oxide synthase (eNOS) expression and phosphorylation1-3; (2) attenuation of vascular oxidative stress by higher local extracellular superoxide dismutase activity4; and (3) collateral formation.5 Traditionally, collateralization has been viewed as a recruitment of preexisting collaterals (arteriogenesis) or an expansion of microvessels (angiogenesis) into ischemic areas. In recent years, this concept has been extended by the observation that postnatal angiogenesis may also be promoted by bone marrow–derived circulating progenitor cells (CPCs), which “home” into ischemic regions and form entirely new vessels by cell division and differentiation into endothelial cells (a process referred to as vasculogenesis).6 In clinical and experimental situations associated with prolonged tissue ischemia (eg, myocardial infarction, hindlimb ischemia), the number of CPCs is significantly increased.7-9 Besides their contribution to vasculogenesis, CPCs might also improve endothelial function of existing arteries by integrating into defects of the endothelial cell layer that are characteristic of atherosclerotic lesions.

As recently published, the amount of CPCs increases significantly even after 7 days of ET in wild-type mice. The CPC rise was absent in eNOS-knockout mice and in wild-
type mice treated with the NOS inhibitor nitro-L-arginine methyl ester. These data suggest a key role for NO in CPC mobilization by regular ET, at least in this animal model.

Lately, we were able to show that 4 weeks of aerobic, subischemic ET in patients with coronary artery disease (CAD) resulted in an increase in vascular eNOS expression, AKT-mediated eNOS phosphorylation, and consequently, NO production, as evidenced by enhanced endothelium-dependent vasorelaxation. However, it is unknown whether the aforementioned training-induced increase in NO production in patients with ischemic syndromes is linked to a CPC release from the bone marrow. In addition to NO, brief reversible episodes of ischemia have been suggested as a stimulus for a vascular endothelial growth factor (VEGF)–mediated release of CPCs from the bone marrow in patients with CAD. Therefore, it was the aim of the 3 present prospective, randomized trials to determine whether brief episodes of ischemia are required during ET-interventions in patients with ischemic syndromes to increase the CPC count and promote homing of CPCs into vascular networks.

Methods

Patient Characteristics

Study Group A
Patients with angiographically documented peripheral arterial occlusive disease (PAOD; age <75 years, Fontaine IIb) were enrolled in the ischemic training study.

Study Group B
Patients with PAOD (age <75 years, Fontaine IIb) after successful revascularization were enrolled in the nonischemic training study. Documented CAD, isolated lower-limb PAOD, Leriche syndrome, and other noncardiac exercise-limiting conditions (orthopedic or pulmonary disorders) were regarded as exclusion criteria for study groups A and B.

Study Group C
For the subischemic training arm of the study, patients with stable CAD (age <75 years) and a minimal symptom-free exercise tolerance of 75 W were recruited. Patients with myocardial infarction within the last 4 weeks, PAOD, left main coronary artery stenosis, documented ventricular arrhythmias (≥Lown IVb), significant valvular heart disease, insulin-dependent diabetes mellitus, and indication for urgent surgical revascularization were excluded from study participation.

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 at the beginning of the study.

Study Design
Patients in study groups A, B, and C were prospectively randomized either to a 4-week training intervention or to usual care. The details of the training programs are reported later. Patients in the usual-care group were followed up by their private physicians and received standard medical treatment. They were advised to continue their previous lifestyle and physical activity.

Study Group A: Ischemic Treadmill Training
Patients with PAOD first underwent a maximal treadmill test until they complained about ischemic leg pain. The presence of ischemia was confirmed by the absence of any change in venous lactate levels. Blood samples were obtained to measure CPCs and VEGF levels, and the number of CPCs was analyzed by flow cytometry. After normalization of plasma VEGF and CPC counts, patients were randomized to the training or control group.

Patients in the training group performed treadmill training 6 times daily for 5 days per week for a period of 4 weeks on a calibrated, electronically braked treadmill with an inclination of 12% and a speed of 3.5 km/h. Patients exercised at 75% of their maximum walking distance of the initial treadmill test. After a break of 1 to 2 minutes, exercise was continued for a second time, followed by a break of at least 1 hour.

At baseline and after 4 weeks, the ABI was assessed by a technician blinded to patient status and group assignment. In addition, the pain-free and the maximal walking distances were both determined on a treadmill set at a speed of 3.5 km/h. At baseline and after 4 weeks, blood lactate was measured to confirm ischemia during the maximal walking test. To rule out an effect of short-term exercise on VEGF and CPC counts, blood samples were always taken after a weekend of physical inactivity (≥72-hour resting period).

Study Group B: Nonischemic Treadmill Training
Patients with successfully treated PAOD first underwent a maximal treadmill test until they stopped because of dyspnea or peripheral exhaustion. The absence of ischemia was documented by the absence of change in venous lactate levels. Blood samples were obtained to measure CPCs and VEGF levels before the treadmill test. After baseline assessment, patients were randomized to the training or control group.

Patients in the training group performed treadmill training 6 times daily for 5 days per week for a period of 4 weeks on a calibrated, electronically braked treadmill with an inclination of 12% and a speed of 3.5 km/h. Patients exercised at 75% of their maximum walking distance of the initial treadmill test. After a break of 1 to 2 minutes, exercise was continued for a second time, followed by a break of at least 1 hour.

At baseline and after 4 weeks, the ABI was assessed by a technician blinded to patient status and group assignment. In addition, the pain-free and the maximal walking distances were determined at study beginning and after 4 weeks via a treadmill set at a speed of 3.5 km/h. At baseline and after 4 weeks, blood lactate was measured to confirm the absence of ischemia during the maximal walking test. To rule out an effect of short-term exercise on VEGF and CPC counts, blood samples were always taken after a weekend of physical inactivity (≥72-hour resting period).

Study Group C: Subischemic Ergometer Training
In patients with stable CAD, maximal symptom-limited ergospirometry was performed as previously described. Myocardial ischemia was confirmed by the presence of typical angina pectoris or significant exercise-induced ST-segment depression (≥0.1 mV descending or horizontal). Patients were randomized either to training or to a control group. After 4 weeks, the symptom-limited maximal exercise test was repeated.

CAD patients randomized to the training group were expected to exercise under close supervision 6 times per day for 10 minutes on a bicycle ergometer below the angina pectoris threshold at 70% of the heart rate reached at peak oxygen uptake during the initial study (excluding a 5-minute warming-up and cooling-down period during each session) for 5 days per week. At this workload, no patient had either angina pectoris symptoms or ST-segment changes. Patients assigned to the control group received their previous medication, continued their sedentary lifestyle, and were supervised by their physicians. Blood samples were always taken after a weekend of physical inactivity (≥72-hour resting period).

Flow Cytometry Analysis
At baseline and after 1, 2, 3, and 4 weeks, venous blood samples were obtained, and the number of CPCs was analyzed by fluorescence-activated cell sorting (FACS) with the following antibodies: anti-human KDR (R&D Systems) and anti-human CD34 (BD Biosiences). The variability of FACS measurements was <0.5% for single-positive events and <1% for double-positive events.
Isolation, Culture, and Characterization of CPCs
At the beginning and after 1, 2, 3, and 4 weeks, venous blood samples were taken. Mononuclear cells were isolated by density-gradient centrifugation from 20 mL of peripheral blood and cultured for 4 days, and the amount of adherent cells doubly positive for 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-LDL uptake and fluorescein isothiocyanate (FITC)-lectin staining was quantified by laser scanning cytometry as recently described.

VEGF, bFGF, GM-CSF, and TNF-α
Plasma levels of VEGF, basic fibroblast growth factor (bFGF), granulocyte monocyte–colony-stimulating factor (GM-CSF), and tumor necrosis factor-α (TNF-α) were measured by highly sensitive ELISAs (R&D Systems) according to the manufacturer’s instructions. Results were compared with standard curves, and the lower detection limits were as follows: for VEGF, 9 pg/mL; GM-CSF, 0.4 pg/mL; TNF-α, 0.18 pg/mL; and bFGF, 0.22 pg/mL. The intra-assay and interassay variabilities were both <10%. Measurements were performed in duplicate.

Matrigel Assay
CPCs and human coronary artery endothelial cells were labeled with Dil–acetylated LDL and FITC-lectin, respectively, and harvested by trypsinization. Human coronary artery endothelial cells (10⁵) were seeded with 10⁵ CPCs on the surface of the extracellular matrix gel (Chemicon) in 8-well chamber slides (Nunc) and incubated at 37°C for 16 hours. After a thorough wash with phosphate-buffered saline, nuclei were stained with Hoechst 33342 (Sigma), and the number of CPCs and human coronary artery endothelial cells per structure was counted in 3 randomly chosen microscopic fields per sample by 2 independent investigators.

Reverse-Transcription–Polymerase Chain Reaction
Total mRNA was isolated from CPCs after 4 days in culture. mRNA expression of CXCR4 and VLA4 was analyzed by real-time reverse-transcription (RT)-polymerase chain reaction (PCR) with specific primers: for CXCR4 forward, 5'-CTGAGAAGCATGACGGACAA-3' and reverse, 5'-TGGAGTGTGACAGCTTGGAG-3'; and for VLA4 forward, 5'-CTGCAATGCAGACGTTGA-3' and reverse, 5'-TTGATTTGGCTCTGGAAAAC-3'); these were normalized to 18S rRNA (forward, 5'-TAGAGGGACAAGTGGCGTTC-3' and reverse, 5'-TGTAACAGGGACGGTACTT-3').

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 2-way repeated-measures ANOVA followed by the Tukey post hoc test, a Mann-Whitney U test, or a Wilcoxon signed-rank test when appropriate. Intrigroup compari-

Figure 1. Quantitative evaluation of CPCs by FACS analysis. Amount of CPCs in blood samples of training (circles) and control (triangles) patients with ischemic PAOD (A), patients with nonischemic PAOD (B), and patients with CAD (C) was analyzed weekly after resting period of at least 72 hours. Representative FACS analysis, in which CD34/KDR cells were gated and further analyzed for CD34/KDR cells (demonstrating differences between baseline and after 4 weeks of training) in patient with ischemic PAOD is shown at top of figure. Values are expressed as fold increase vs baseline and are mean±SEM. *P<0.05 vs control.
sions were performed with a nonparametric test (Wilcoxon test). A probability value <0.05 was considered statistically significant.

Results

Patient Characteristics

Study Group A
A total of 18 patients with stable PAOD (Fontaine IIb; mean±SEM age, 57±2 years; mean±SEM body mass index, 27±1 kg/m²) for whom interventional revascularization was recommended were enrolled in the study and randomized to a training or a control group. Both groups did not differ with respect to any of the baseline parameters (see online-only Data Supplement).

Study Group B
A total of 18 patients with prior PAOD (Fontaine IIb; mean±SEM age, 63±7 years; mean±SEM body mass index, 27±2 kg/m²) who were interventional revascularized were enrolled in the study and randomized to a training or a control group. Both groups did not differ with respect to any of the baseline parameters (see Data Supplement).

Study Group C
For the subschematic training arm, a total of 31 patients with stable CAD (mean±SEM age, 61±2 years; mean±SEM body mass index, 27±1 kg/m²) were randomized to a training or a control group. There were no differences with regard to baseline parameters between patients in the training and control group (see Data Supplement).

Evidence of Ischemia in Patients With PAOD and CAD

Study Group A
In patients with ischemic PAOD, the average initial duration of ischemia (calculated on reported ischemic leg pain) was 9.4±2.1 minutes, which was confirmed by an increase in the postexercise lactate value of 3.3±1.7 mmol/L (from 0.2±0.1 mmol/L to 3.5±1.7 mmol/L, P<0.05 versus baseline) after the initial exercise test.

Study Group B
In patients with nonischemic PAOD, no significant exercise-induced ischemic leg pain was detected. The absence of ischemia was confirmed by unchanged postexercise lactate values (from 0.9±0.1 mmol/L to 1.1±0.3 mmol/L, P=NS versus baseline) after the initial exercise test.

Study Group C
No significant exercise-induced ST-segment depressions or presence of typical angina pectoris were observed in the CAD group during the training sessions.

Effects of ET on Clinical Parameters in Patients With PAOD and CAD

Study Group A
Patients in the ET group showed significant improvement in symptom-free and maximal walking distances, whereas both parameters remained virtually unchanged in the control group (see Data Supplement).

Effects of ET on CPC Levels, Cytokine Concentrations, and CPC Function

Effects of ET on CPC Levels
Ischemic walking training (group A) led to a significant and time-dependent, 5.2-fold increase in the fraction of CD34+/KDR+ CPCs (Figure 1A and Table 1). This increase, as documented by FACS analysis, was confirmed in cell culture experiments (Figure 2A and Table 1). On the other hand, subschematic ET in patients with PAOD or CAD (groups B or C) did not result in any increase in CPCs as analyzed by FACS (Figures 1B, 1C, 2B, and 2C and Tables 2 and 3). In addition, the amount of CPCs did not change in the control group during the training sessions.

By Doppler ultrasound measurements, the ABI increased in the training group by 33% after 4 weeks (0.6±0.1 at baseline versus 0.8±0.1 at 4 weeks, P<0.05 versus control), whereas no change was observed in the control group (0.6±0.1 at baseline versus 0.6±0.1 at 4 weeks). Two patients in the training group declined the scheduled interventional treatment of their PAOD after 4 weeks of limb training because of complete resolution of subjective walking-induced symptoms.

Study Group B
Patients in the ET group were characterized by an increase in maximal walking distance, whereas this parameter remained virtually unchanged in the control group (see Data Supplement).

Study Group C
ET resulted in a significant improvement of exercise capacity (132±7 W at baseline versus 156±11 W at 4 weeks, P<0.05 versus control) and in VO2max (19.5±2.5 mL·kg⁻¹·min⁻¹ at baseline to 22.1±3.4 mL·kg⁻¹·min⁻¹ at 4 weeks, P<0.05 versus control and baseline). The ischemic threshold for patients in the ET group increased from 96±8 W at baseline to 129±10 W at 4 weeks (P<0.05 versus control and baseline). All of these parameters were unaffected in the control group (see Data Supplement).

Effects of ET on CPC Levels

TABLE 1. Amount of CPCs, Cytokines, Integrative Capacity, and Homing Factors in Study Group A: Patients With Ischemic PAOD

<table>
<thead>
<tr>
<th>Training (n=9)</th>
<th>Control (n=9)</th>
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<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td><strong>4 Weeks</strong></td>
</tr>
<tr>
<td>CD34+/CD34⁻ cells</td>
<td>458±84</td>
</tr>
<tr>
<td>CD34+/KDR+ cells</td>
<td>90±14</td>
</tr>
<tr>
<td>Dil-LDL/lectin⁻ cells</td>
<td>1094±26</td>
</tr>
<tr>
<td>VEGF</td>
<td>17.5±3.4</td>
</tr>
<tr>
<td>Integrative capacity</td>
<td>7.5±4.7</td>
</tr>
<tr>
<td>CXCR4</td>
<td>2.8±1.1</td>
</tr>
<tr>
<td>VLA4</td>
<td>3.2±2.3</td>
</tr>
</tbody>
</table>

Data are shown as mean±SEM. Amounts of CD34+/CD34⁻ cells and CD34+/KDR+ cells are expressed as cells per mL blood. Amounts of Dil-LDL/lectin⁻ cells are expressed as cells per well. Cytokine concentrations are expressed as pg/mL, integrative capacity as % integrated CPCs, and homing factors as arbitrary units mRNA.

*P<0.05 vs beginning; †P<0.05 vs control after 4 weeks.
groups during the study period of 4 weeks (Figures 1 and 2 and Table 1).

**Effects of ET on CPC Function**

In group A, to assess the influence of ischemic ET on CPC function, the capacity of CPCs to participate in network formation was analyzed by a Matrigel assay. Ischemic walking training led to a significant enhancement of CPCs integrating into endothelial networks (7.5 ± 4.7% at baseline versus 19.5 ± 7.7% integrated CPCs at 4 weeks, *P* < 0.05 versus control and baseline), whereas in the inactive control group, no change could be documented (9.2 ± 5.9% at baseline).

**TABLE 2. Amount of CPCs, Cytokines, Integrative Capacity, and Homing Factors in Study Group B: Patients With Nonischemic PAOD**

<table>
<thead>
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<th>Training (n=9)</th>
<th>Control (n=9)</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>4 Weeks</td>
</tr>
<tr>
<td>CD34⁺ cells</td>
<td>425 ± 48</td>
<td>419 ± 59</td>
</tr>
<tr>
<td>CD34⁺/KDR cells</td>
<td>74 ± 13</td>
<td>94 ± 17</td>
</tr>
<tr>
<td>Dil-LDL/lectin⁺ cells</td>
<td>752 ± 72</td>
<td>971 ± 31</td>
</tr>
<tr>
<td>VEGF</td>
<td>21.4 ± 3.4</td>
<td>15.3 ± 5.1</td>
</tr>
<tr>
<td>Integrative capacity</td>
<td>7.9 ± 2.4</td>
<td>8.5 ± 4.1</td>
</tr>
<tr>
<td>CXCR4</td>
<td>3.9 ± 1.1</td>
<td>4.6 ± 1.9</td>
</tr>
<tr>
<td>VLA4</td>
<td>4.2 ± 1.2</td>
<td>4.8 ± 2.5</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM. Amounts of CD34⁺ cells and CD 34⁺/KDR⁺ cells are expressed as cells per mL blood. Amounts of Dil-LDL⁺/lectin⁺ cells are expressed as cells per well. Cytokine concentrations are expressed as pg/mL, integrative capacity as % integrated CPCs, and homing factors as arbitrary units mRNA.

*P* < 0.05 vs beginning; †*P* < 0.05 vs control after 4 weeks.

**TABLE 3. Amount of CPCs, Cytokines, Integrative Capacity, and Homing Factors in Study Group C: Patients With CAD**

<table>
<thead>
<tr>
<th></th>
<th>Training (n=15)</th>
<th>Control (n=16)</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>4 Weeks</td>
</tr>
<tr>
<td>CD34⁺ cells</td>
<td>582 ± 46</td>
<td>549 ± 67</td>
</tr>
<tr>
<td>CD34⁺/KDR⁺ cells</td>
<td>110 ± 16</td>
<td>126 ± 15</td>
</tr>
<tr>
<td>Dil-LDL⁺/lectin⁺ cells</td>
<td>975 ± 28</td>
<td>958 ± 35</td>
</tr>
<tr>
<td>VEGF</td>
<td>20.5 ± 5.9</td>
<td>22.8 ± 7.1</td>
</tr>
<tr>
<td>Integrative capacity</td>
<td>6.8 ± 3.9</td>
<td>21.7 ± 9.2†</td>
</tr>
<tr>
<td>CXCR4</td>
<td>2.8 ± 1.6</td>
<td>6.2 ± 1.9†</td>
</tr>
<tr>
<td>VLA4</td>
<td>3.6 ± 1.5</td>
<td>7.4 ± 2.8†</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM. Amounts of CD34⁺ cells and CD 34⁺/KDR⁺ cells are expressed as cells per mL blood. Amounts of Dil-LDL⁺/lectin⁺ cells are expressed as cells per well. Cytokine concentrations are expressed as pg/mL, integrative capacity as % integrated CPCs, and homing factors as arbitrary units mRNA.

*P* < 0.05 vs beginning; †*P* < 0.05 vs control after 4 weeks.
line versus 10.3±5.9% integrated CPCs at 4 weeks, P=NS; Figure 4A and Table 1). The improved ability of CPCs to incorporate into an endothelial network was accompanied by a significantly higher expression of CXCR4 (+107%, Table 1). With respect to the aforementioned parameters, no change was observed in the control group.

In groups B and C, there was no effect on CPC count in the blood, but the integrative capacity of CPCs was significantly increased (PAOD, 7.9±2.4 at baseline versus 24.1±9.2% after 4 weeks, P<0.05 versus control and baseline; CAD, 6.8±3.9 at baseline versus 21.7±9.2% after 4 weeks, P<0.05 versus control and baseline) (Figure 4B and 4C and Table 1). This was accompanied by a rise in mRNA expression of CXCR4 (group B, +277%; group C, +121%) and VLA4 (group B, +61%; group C, +105%; Table 1). Improvement in functional capacity was correlated positively with the changes in CXCR4 mRNA expression in PAOD training patients (r=0.51, P<0.05) and CAD training patients (r=0.56, P<0.05). In the respective control groups, no change was observed with respect to the integrative capacity and the expression of CXCR4 and VLA4 (Table 1).

Effects of ET on Plasma Concentrations of VEGF, GM-CSF, bFGF, and TNF-α
In the ischemic ET group (A), plasma VEGF concentration rose by 103±26% as a result of 2 weeks of ET (17.5±3.4 pg/mL at baseline versus 35.5±4.1 pg/mL after 2 weeks, P<0.05 versus control). However, an even longer duration of training was linked to a further increase in plasma VEGF concentration (73.2±5.8 pg/mL at 4 weeks, P<0.05 versus control and baseline; Figure 3A and Table 1). In contrast, VEGF concentration did not change in the control group. Furthermore, changes in VEGF concentrations exhibited a linear correlation with changes in the number of CPCs...
VEGF, GM-CSF, TNF-α, or bFGF, no significant alterations were observed in the training or control-group (data not shown).

In groups B and C, in contrast, subischemic ET in patients with PAOD and CAD for a period of 4 weeks did not affect VEGF, GM-CSF, TNF-α, or bFGF plasma levels (data not shown).

Discussion
In these prospective, randomized, clinical trials, we assessed the effects of ischemic and nonischemic walking training in PAOD and subischemic ET in stable CAD on the number and in vitro function of CPCs. The following key inferences are supported by our findings: (1) Symptomatic ischemia seems to be a necessary trigger for CPC release and mobilization into the peripheral circulation. (2) The release of CPCs is associated with an increase in plasma VEGF concentrations, and a correlation between changes in VEGF and changes in CPC levels suggests that an increase in VEGF might account for CPC mobilization. (3) Both subischemic and ischemic ET led to an improved CPC incorporation into vascular structures, most likely due to the induced expression of homing factors like CXCR4 and VLA 4.

Symptomatic Ischemia as a Trigger for CPC Release
Data from animal experiments suggest that an ischemic stimulus is able to induce a significant release of CPCs from the bone marrow.19 Those cells were recently described to home into ischemic tissues and promote neovascularization.9,17 In the present study, we were able to document that repetitive episodes of ischemia during ET in patients with PAOD are required to induce a sustained elevation of plasma VEGF concentrations and CPC counts. Despite the accumulating consensus that CPC release constitutes a physiologically relevant reaction of the organism to tissue ischemia, controversies about the exact mediators for CPC mobilization exist.

Cytokines
Asahara et al8 reported that hematopoietic/angiogenic cytokines (eg, VEGF and GM-CSF) mobilize CPCs in animal models. The pathophysiological role of VEGF was further elucidated by the finding of Shintani et al7 that only VEGF was significantly elevated in post–myocardial infarction patients. Although the exact origin of VEGF production has not been elucidated yet, it is well known that the promoter sequence of VEGF contains hypoxia-responsive elements and that intramyocardial VEGF concentrations are elevated after acute myocardial infarction.20 In the study of Shintani et al, a close correlation between plasma VEGF levels and CPCs was observed, a finding confirmed in the present trial.

Even after a single episode of short-term exercise–induced ischemia in patients with stable CAD, a significant rise in VEGF and CPCs was detectable.14 Our findings of increased plasma VEGF levels and consecutively elevated CPC numbers in PAOD patients exposed to repetitive ischemia are consistent with these previously published data and confirm the role for VEGF in CPC mobilization. We have recently documented that bFGF, GM-CSF, nor TNF-α is involved in ischemia-induced mobilization of CPCs.14 The present study confirms the lack of any significant role for these mediators.

Nitric Oxide
Two previous studies described a close dependence of CPC mobilization on NO generation. Aicher and colleagues21 showed that eNOS−/− mice exhibit reduced VEGF-induced mobilization of CPCs. Intravenous infusion of wild-type PCs rescued the defective neovascularization of eNOS−/− mice in a model of hindlimb ischemia. Recently, Laufs and colleagues10 measured the increase in circulating CPCs after ET in wild-type and eNOS−/− mice. Because changes in CPC count were absent in eNOS−/− mice in response to exercise, those authors hypothesized that NO would be an important mediator for CPC release.

We have previously documented that endothelial function is systemically improved after systemic ET in patients with CAD by flow-mediated increases of eNOS expression and activity,3 leading to an increased NO bioavailability. In the present trial, patients with CAD who met the inclusion criteria of the study exercised under the same condition. Therefore, it can be assumed that eNOS expression and NO production were enhanced in the peripheral vasculature and perhaps in vessels supplying blood to the bone marrow in this population of CAD patients after ET. Although NO production per se was not measured in the present study, we cannot rule out that an increase in NO production alone is insufficient to augment CPC counts in patients with CAD undergoing daily subischemic ET.

So far, only limited data are available on the interaction between exercise and CPC release. Laufs and colleagues10 described a significant increase in CPCs after a 4-week, noncontrolled rehabilitation training program in patients with stable CAD without exercise-induced ischemia. However, the reported baseline VO2max of 9.6 ± 4.0 mL · kg−1 · min−1 is more plausible for patients in advanced chronic heart failure, in which condition both endothelial dysfunction and reduced cardiac output lead to peripheral hypoperfusion during exercise.22,23 It can be speculated that asymptomatic tissue ischemia leads to an increase in vasculogenic cytokines, like symptomatic tissue ischemia does.24 Another important difference between the present trial and the study of Laufs et al concerns the training program. We strictly used aerobic submaximal endurance training on a bicycle ergometer, whereas Laufs et al combined high-intensity ergometer training at 60% to 80% of VO2max with moderate muscle strength training. Resistance exercise is known to lead to repetitive reductions in muscle perfusion due to compression of arterial vessels. This could further deteriorate peripheral perfusion in a population of patients with presumably subnormal left ventricular function.

Furthermore, the markers used to detect CPCs were different between the studies. Laufs and colleagues used sca-1/flk-1 to define CPCs in mice, whereas in the present study, only cells doubly positive for CD34 and KDR were counted as CPCs. This difference may be important, because it is known that sca-1 is not exclusively expressed on PCs but may also be present on mature endothelial cells.25

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(r=0.66, P<0.05; Figure 3D). For GM-CSF, TNF-α, or bFGF, no significant alterations were observed in the training or control-group (data not shown).
It is important to note that a rise in CPCs did not occur as a specific response but rather in the context of a significant increase in CD34-positive cells. In ischemic PAOD patients, the 440% rise in CPCs was accompanied by a 550% increase in CD34-positive cells. This observation suggests that the response of the bone marrow to episodes of ischemia is not limited to CPC release but constitutes a complex reaction, with different PC populations and leukocyte subtypes being involved.

**Improvement of CPC Function After ET**

It has become clear that not only the number of CPCs but also their function is modified by pathological conditions and therapeutic interventions. Adhesion of CPCs to activated endothelial cells and integration into the extracellular matrix is believed to be important during growth of new blood-vessels.28

In the present study, a Matrigel assay was performed to investigate the ability of CPCs to integrate into vascular structures. Cocultures of CPCs and human coronary artery endothelial cells on Matrigel led to the formation of a more extensive tubule network in PAOD patients after both ischemic and nonischemic walking training and in CAD patients after subischemic ergometer training. This finding is consistent with the notion that VEGF, which was differentially regulated in ischemic and subischemic training, is most likely not involved in modification of the functional characteristics of CPCs. Upregulation of CXCR4 and VLA4 as homing markers of PCs29 in patients after ischemic and subischemic training provides an argument for improved integrative capacity in response to ET. This notion is further supported by the observation that mice lacking CXCR4 die in utero because of defects in vascular development.30 The upregulation of CXCR4 is possibly mediated by a cAMP-responsive element31 in the promotor region of the gene. cAMP is known to be increased during ischemic and subischemic ET.32

Although 4 weeks of subischemic training failed to mobilize CPCs, ET has well-documented positive effects on coronary vasomotion and myocardial perfusion. The lack of significant CPC mobilization suggests that subischemic training primarily exerts its effects by beneficial shear stress–mediated improvements in local vascular NO availability and antioxidative protection. An improved ability of CPCs to incorporate into vascular structures might also be a further explanation for these positive effects in response to ET.

**Conclusion**

In this first prospective, randomized, clinical study, we confirmed the obligatory role of symptomatic ischemia for the increase in CPC count and VEGF release in patients with ischemic vascular syndromes. However, both ischemic and nonischemic walking training programs in PAOD as well as subischemic ergometer training in CAD improved CPC function as assessed by Matrigel assay.

Two major conclusions may be drawn from these findings: (1) Tissue ischemia seems to be a prerequisite for VEGF release and consecutive CPC release, and (2) mediators besides VEGF are involved in the regulation of the integrative capacity of CPCs.

The 3 presented randomized trails demonstrate that ET in cardiovascular diseases represents an effective stimulus to enhance the regenerative capacity with regard to CPCs, which may in fact decelerate disease progression.

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