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Exercise Acutely Increases Circulating Endothelial Progenitor Cells and Monocyte-/Macrophage-Derived Angiogenic Cells

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OBJECTIVES	We investigated whether a single episode of exercise could acutely increase the numbers of endothelial progenitor cells (EPCs) and cultured/circulating angiogenic cells (CACs) in human subjects.
BACKGROUND	Endothelial progenitor cells and CACs can be isolated from peripheral blood and have been shown to participate in vascular repair and angiogenesis. We hypothesized that exercise may acutely increase either circulating EPCs or CACs.
METHODS	Volunteer subjects (n = 22) underwent exhaustive dynamic exercise. Blood was drawn before and after exercise, and circulating EPC numbers as well as plasma levels of angiogenic growth factors were assessed. The CACs were obtained by culturing mononuclear cells and the secretion of multiple angiogenic growth factors by CACs was determined.
RESULTS	Circulating EPCs (AC133+/VE-Cadherin+ cells) increased nearly four-fold in peripheral blood from 66 ± 27 cells/ml to 236 ± 34 cells/ml ($p < 0.05$). The number of isolated CACs increased 2.5-fold from $8,754 \pm 2,048$ cells/ml of peripheral blood to $20,759 \pm 4,676$ cells/ml ($p < 0.005$). Cultured angiogenic cells isolated before and after exercise showed similar secretion patterns of angiogenic growth factors.
CONCLUSIONS	Our study demonstrates that exercise can acutely increase EPCs and CACs. Given the ability of these cell populations to promote angiogenesis and vascular regeneration, the exercise-induced cell mobilization may serve as a physiologic repair or compensation mechanism. (J Am Coll Cardiol 2004;43:2314–8) © 2004 by the American College of Cardiology Foundation

Endothelial progenitor cells (EPCs) have recently been identified as a circulating cell population in peripheral blood (1). Such EPCs co-express hematopoietic stem/progenitor cell markers (CD34 or AC133) as well as endothelial markers (VE-Cadherin or VEGFR-2) (1–3). When transplanted into animal models, EPCs appear to incorporate into sites of neovascularization and promote angiogenesis (1,4). Endothelial progenitor cells are rare in circulation but can be mobilized into circulation from bone marrow by vascular trauma or systemic administration of cytokines (3,5). In addition to their angiogenic effects, EPCs may also serve as “repair cells” that can replace dysfunctional endothelium (6).

In distinction from circulating EPCs defined by specific stem/progenitor cell markers, culturing of mononuclear cells (MNCs) on fibronectin yields a population that exhibits certain endothelial characteristics and can enhance angiogenesis following transplantation of such cells (7). The vast majority of these cells express monocyte/macrophage mark-

ers and secrete the angiogenic growth factors vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) (8). This cell population has been recently redefined as CACs (circulating angiogenic cells or cultured angiogenic cells) (8) to distinguish the cells from EPCs, as the latter do not appear to express monocyte/macrophage markers (3).

The number of both cell populations can be increased by pharmacologic therapy with HMG-CoA-reductase inhibitors (9). Less is known about whether the circulating levels of these cell populations can be increased by nonpharmacologic interventions. Exercise is a nonpharmacologic intervention that can acutely mobilize specific MNC subsets into circulation (10). We therefore investigated the effects of acute exercise on circulating EPCs and CACs and were able to demonstrate that both cell populations increase acutely following exercise.

METHODS

Study protocol. The protocol was approved by the Institutional Review Board of the Indiana University School of Medicine. We enrolled 22 volunteer patients (Table 1) who provided informed consent. After a pre-exercise blood sample was drawn in the supine position, patients under-

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Abbreviations and Acronyms

CAC	= cultured/circulating angiogenic cell
EPC	= endothelial progenitor cell
G-CSF	= granulocyte colony-stimulating factor
GM-CSF	= granulocyte macrophage-colony stimulating factor
HGF	= hepatocyte growth factor
LDL	= low-density lipoprotein
MNC	= mononuclear cells
VEGF	= vascular endothelial growth factor

went a symptom-limited treadmill or bicycle exercise test. Post-exercise blood samples were drawn within 5 to 10 min after completion of exercise while the patient was in a supine position. Blood samples were used for a complete blood count (n = 21), circulating progenitor cell analysis (n = 10), MNC culture (n = 18), and plasma collection (n = 14). All patients were thought to have a negative stress test by their electrocardiographic and echocardiographic assessment as per the supervising cardiology physician blinded to our cell analysis.

Identification of circulating EPCs and hematopoietic stem/progenitor cells. For flow cytometric determination of circulating endothelial progenitor cells (AC133+/VE-Cadherin+) or hematopoietic stem/progenitor cells (AC133+/VE-Cadherin-), 100 ml of whole blood was labeled for 20 to 30 min at room temperature using manufacturer-recommended concentrations with antihuman-VE-Cadherin-PE (Becton Dickinson, San Diego, California) and antihuman-AC133-APC (Miltenyi Biotec, Auburn, California). Fluorescent isotype-matched antibodies (Becton Dickinson, San Diego, California) were used as controls. The suspension was then incubated with FACS lysing solution (Becton Dickinson) according to manufacturer instructions for 10 to 15 min. After washing and fixation, samples were analyzed on a FACS-Calibur Instrument (Becton-Dickinson, San Jose, California). Surface

Table 1. Summary of Clinical and Exercise Data of Our Study Cohort (n = 22)

Mean age (yrs)	54 ± 10
Men	16 (72%)
Diabetes	2 (9%)
Hypertension	11 (50%)
Hyperlipidemia	9 (40%)
Smoking	9 (40%)
Family history of premature CAD	10 (45%)
No. of patients on statins	4 (18%)
Body mass index	29 ± 1
Pre-exercise resting heart rate (beats/min)	75 ± 13
Postexercise maximum heart rate (beats/min)	150 ± 19
Peak exercise double product (peak systolic blood pressure × peak heart rate)	29,033 ± 6,099
Maximum mean exercise workload (METS)	9.5 ± 2.4
Percentage of age predicted maximum heart rate (peak heart rate/220 – age × 100%)	90 ± 9

CAD = coronary artery disease; METS = metabolic equivalents.

markers AC133 (also known as CD133) and VE-Cadherin were determined on cells in the lymphocyte gate, because this is where EPCs are commonly found (11). The percentages of positive cells were converted to cells per ml of blood using the complete blood count.

CAC isolation and characterization. Cultured angiogenic cells were obtained as previously described by culturing MNCs in EGM-2-MV medium (Cambrex-Clonetics, Baltimore, Maryland) for four days (8). Adherent cells staining positive for both *Ulex europaeus* agglutinin-I and DiI-acetylated low-density lipoprotein (LDL) were judged as CACs (8). Staining of cell nuclei with DAPI (10 μmol/l for 5 to 10 min) verified that nearly all adherent cells (>95%) before and after exercise were acetylated LDL(+)Ulex-Lectin(+). Therefore, the CAC cell numbers were determined by detaching adherent cells and counting them with a hemacytometer. Flow cytometric analysis of pre- and post-exercise CACs (data not shown) confirmed previous findings that most CACs express monocyte/macrophage markers (8).

Plasma growth factor determination. Plasma was assayed for vascular endothelial growth factor (VEGF), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) using a Luminex analyzer and Multi-Analyte Profiling Fluorokine kits from R&D Systems (Minneapolis, Minnesota) according to manufacturer instructions. Hepatocyte growth factor (HGF) was assayed by ELISA (R&D Systems). Data are expressed as mean ± SEM pg/ml plasma.

CAC paracrine activity. Cultured angiogenic cells obtained before and after exercise were switched to the basal medium EBM-2/5%–fetal bovine serum (no supplemental growth factors) on day 4 for 72 h. Conditioned media were assayed for the growth factors VEGF, HGF, G-CSF, and GM-CSF as previously described. The preconditioned medium did not contain significant amounts of these factors. Data are expressed as mean ± SEM pg factor/10⁶ adherent cells.

Statistical analysis. Statistical analysis was performed using Graphpad Prism (Graphpad, San Diego, California) software. Pre- and post-exercise levels of cells or growth factors were compared using the paired *t* test. A value of *p* < 0.05 was considered significant. Quantitative data are presented as mean ± SEM.

RESULTS

Exercise effects on circulating progenitor cells. Circulating EPCs (positive for AC133 and for VE-Cadherin) increased nearly four-fold in peripheral blood from 66 ± 27 cells/ml to 236 ± 34 cells/ml (Fig. 1A) (*p* < 0.05). Cells positive for AC133 but negative for VE-Cadherin (which are likely to represent hematopoietic stem/progenitor cells) showed an increase in peripheral blood of only 40% (Fig. 1B) (*p* < 0.005), from 1,743 ± 272 cells/ml to 2,455 ± 253 cells/ml, thus resulting in an

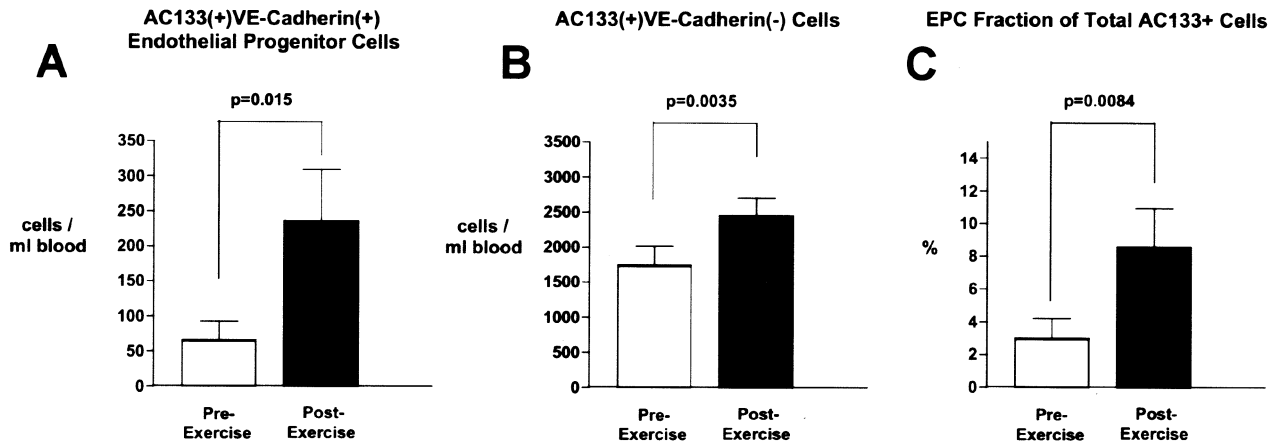


Figure 1. Circulating endothelial progenitor cells (EPCs) were identified in whole blood using the stem/progenitor cell marker AC133 and the endothelial marker VE-Cadherin (A). Non-endothelial stem/progenitor cells also increased (B), but the EPC fraction of AC133+ cells showed the most prominent increase (C). White bars = pre-exercise; black bars = post-exercise. Data are given as mean \pm SEM.

exercise-induced shift toward a greater proportion of circulating progenitor cells expressing the endothelial marker VE-cadherin (Fig. 1C).

Exercise effects on CACs. Cultured angiogenic cells obtained by culturing MNCs from pre-exercise and post-exercise blood samples showed the typical uptake of acetylated LDL and binding of Ulex-lectin (7) (Fig. 2). The number of isolated CACs increased markedly from $8,754 \pm 2,048$ to $20,759 \pm 4,676$ cells/ml of blood ($p < 0.005$) (Fig. 3A). Because exercise increases the number of total circulating MNCs, the number of CACs was normalized per 10^6

isolated MNCs and still showed a significant increase from $7,193 \pm 1,451$ to $10,321 \pm 1,972$ cells/ 10^6 MNCs ($p < 0.005$) (Fig. 3B).

Growth factor secretion by CACs. We investigated the paracrine activity of CACs over a 72-h period in a subset of patients ($n = 6$). As shown in Figure 4, there was a trend toward higher growth factor secretion post-exercise, but it was not statistically significant. This suggests that exercise results in an increased number of CACs are at least equipotent in their ability to release angiogenic growth factors.

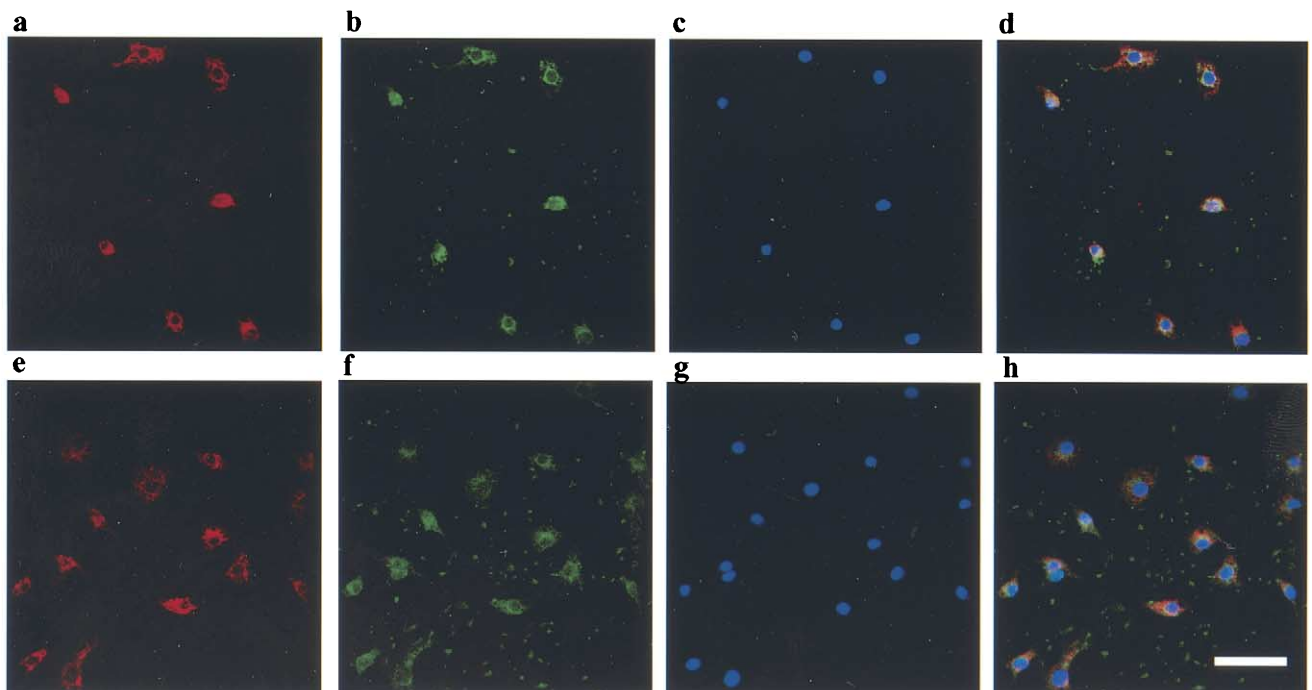


Figure 2. Confocal microscopy (Zeiss LSM 510 UV, 100 \times oil immersion objective) of a representative sample illustrates that pre-exercise adherent cells were positive for the uptake of DiI-labeled acetylated low-density lipoprotein (red) (a) and the binding of FITC-Ulex-Lectin (green) (b). Visualization of nuclei with DAPI (blue) (c) and the overlay of all three fluorescence images (d) demonstrate that all adherent cells fulfill cultured angiogenic cell criteria (acLDL+/Ulex-Lectin+). Respective post-exercise cultured angiogenic cells are shown in the lower panels (e to h). Bar = 25 μ m.

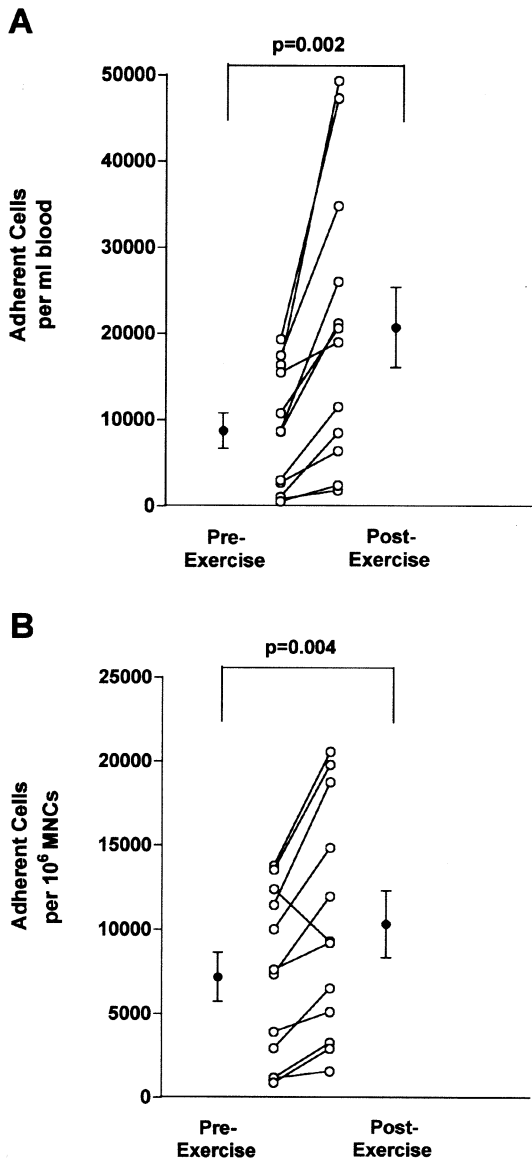


Figure 3. Cultured angiogenic cells were markedly higher per ml of peripheral blood following exercise (A). The increase in cultured angiogenic cells/ 10^6 plated mononuclear cells (MNCs) was also statistically significant (B), thus suggesting that cultured angiogenic cells increase more than overall mononuclear cells following exercise. **White circles** = individual values; **black circles** = mean \pm SEM.

Plasma levels of angiogenic growth factors. Exercise did not markedly change the levels of the assayed growth factors in plasma (VEGF levels increased by 3.3% and HGF levels by 8.8%). We were not able to detect either G-CSF or GM-CSF in pre- and post-exercise plasma.

DISCUSSION

Our study demonstrates for the first time that exercise can acutely increase two distinct cell populations that are known to be involved in angiogenesis and endothelial repair: circulating EPCs, which may supply new endothelial cells to the vasculature (12); and CACs, which secrete growth

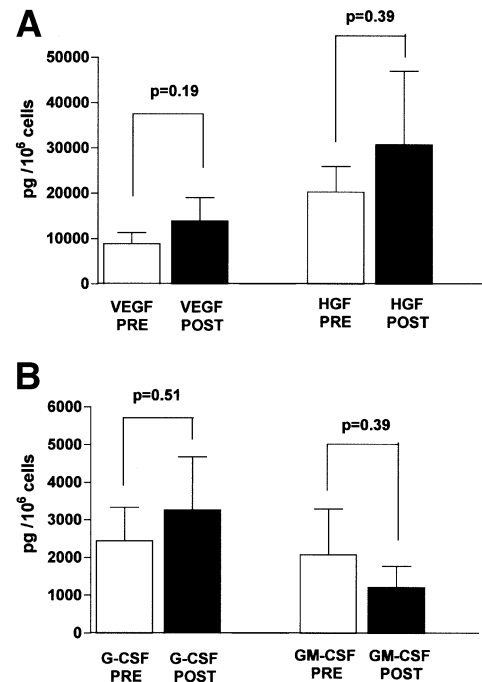


Figure 4. Pre- and post-exercise cultured angiogenic cells showed similar levels of endothelial growth factor secretion (vascular endothelial growth factor [VEGF], hepatocyte growth factor [HGF], A) and colony stimulating factor secretion (granulocyte and granulocyte-macrophage colony stimulating factor [G-CSF, GM-CSF], B) over a 72-h period.

factors that promote endothelial growth and angiogenesis. Additionally, mobilization of non-endothelial AC133+ cells (Fig. 1B), which are likely to be hematopoietic stem/progenitor cells, may explain the previous discovery that exercise can acutely increase the number of peripheral blood colony-forming units (13).

The increase of EPCs and CACs with potentially synergistic functions (supply of “building blocks” and secretion of growth factors respectively) may contribute to exercise-induced angiogenesis (14). These increases in cells are likely to be complementary to the exercise induction of angiogenic growth factors within the muscle tissue (15). Because growth factors such as VEGF can enhance cell migration (11), exercise-induced growth factor expression within the muscle may direct the exercise-mobilized circulating cell populations to migrate into hypoxic muscle tissues.

In addition to the enhancement of angiogenesis, exercise is also known to improve endothelial function (16,17). Recent studies suggest that dysfunctional endothelium can be replenished by bone marrow-derived cells (6). It is therefore possible that, even in the absence of angiogenesis, circulating EPCs and circulating CAC precursors mobilized by exercise may contribute to “endothelial repair” by homing to areas of dysfunctional endothelium and either differentiating into new endothelial cells or secreting vasculoprotective growth factors.

The increase in circulating EPCs within just 10 min of exercise may indicate that this is due either to mobilization

of EPCs from bone marrow or the marginal pool via beta-adrenergic mechanisms, similar to that of lymphocytes (10), or due to exercise-induced changes in shear stress (16), which result in mechanical mobilization of cells. The absence of any detectable increase of G-CSF or GM-CSF in plasma suggests that the mobilization was probably not due to systemic increases in these factors. The increase in CACs, on the other hand, could be due to a variety of factors, given the culture period before their analysis, including mobilization of monocyte subsets or improved CAC survival in culture.

Perspectives and future studies. Our data on exercise modulation of progenitor and pro-angiogenic cells may in part explain the significant cardiovascular benefits of exercise. Future studies with larger patient numbers will be required to investigate whether healthy subjects and specific patient subsets such as obese patients, insulin-dependent diabetics, non-insulin-dependent diabetics, or patients with coronary artery disease demonstrate similar cell mobilization with acute exercise. This may allow for using a nonpharmacologic intervention, such as exercise, to complement pharmacologic interventions such as statin or G-CSF therapy to mobilize EPCs. At the same time, unraveling the mechanisms of exercise-induced EPC mobilization may result in the development of novel pharmacologic pathways to improve endothelial function and enhance angiogenesis.

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