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The Effects of External Counter Pulsation Therapy on Circulating Endothelial Progenitor Cells in Patients with Angina Pectoris

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Key Words
Angina pectoris · External counter pulsation · Endothelial progenitor cells · Ischemia · Stem cells

Abstract
Objectives: External counter pulsation therapy (ECPT) offers symptomatic relief and improves ischemia in patients with refractory angina pectoris. We aimed to determine the effects of ECPT on circulating endothelial progenitor cells (EPCs). ***Methods:*** We prospectively studied 25 patients with angina pectoris treated with ECPT (n = 15) or receiving standard care (n = 10). The number of EPCs positive for CD34 and kinase insert domain receptor (KDR) was determined by flow cytometry and the number of colony-forming units (CFUs) was assessed in a 7-day culture, before ECPT and after 9 weeks. ***Results:*** ECPT improved anginal score from a median of 3.0 to 2.0 ($p < 0.001$). Concomitantly, ECPT increased EPC number from a median of 10.2 to 17.8/10^5 mononuclear cells ($p < 0.05$), and CFUs from 3.5 to 11.0 ($p = 0.01$). Flow-mediated dilatation was improved by ECPT from 7.4 to 12.2% ($p < 0.001$) and correlated with EPC-CFUs ($r = 0.461$, $p = 0.027$). The levels of asymmetric dimethylarginine were reduced by ECPT from 0.70 to 0.60 μmol/l ($p < 0.01$). In contrast, the same parameters did not change in the control group, before and after follow-up. ***Conclusions:*** The present pilot study shows, for the first time, that ECPT is associated with increased number and colony-forming capacity of circulating EPCs.

Introduction

External counter pulsation therapy (ECPT) provides noninvasive treatment for patients suffering from angina pectoris [1]. This technique uses a series of inflatable cuffs placed around the patient's legs. Inflation and deflation of the cuffs is synchronized with the patient's electrocardiogram to provide an effect similar to intraaortic balloon counter pulsation by improving coronary flow and reducing afterload [1, 2]. Prospective clinical trials and data from registry studies have shown that ECPT reduces anginal symptoms, improves exercise capacity and quality of life, extends the time to ischemia on exercise treadmill test, decreases reversible perfusion defects on thallium imaging and improves left ventricular function [3–7]. ECPT has become a viable alternative treatment for patients with refractory angina unsuitable for coronary artery revascularization procedures. However, its specific mechanism of action is unclear [1]. Possible mecha-

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nisms are diastolic augmentation and increased coronary blood flow, decreased vascular resistance and afterload reduction [2], improved endothelial function [8, 9], and new vessel formation [10].

Recently, particular attention has been given to the role of circulating endothelial progenitor cells (EPCs) in the pathobiology of atherosclerosis and neovascularization of ischemic tissue [11, 12]. The number of circulating EPCs has been suggested to mirror vascular health and represent a marker of cardiovascular disease risk [11, 13]. Furthermore, injection of EPCs promotes neovascularization and healing after ischemia and infarction [14, 15]. Thus, we raised the hypothesis that some of the beneficial effects of ECPT may be attributed to the effect on circulating EPCs. The aim of the present study was, therefore, to test this hypothesis in patients with angina pectoris with and without ECPT.

Methods

Study Subjects

This study enrolled 25 patients with symptomatic coronary artery disease (CAD) who were referred for a supervised ECPT program at the Heart Institute of the Sheba Medical Center. Fifteen of these patients were treated with ECPT, while the control group comprised 10 age- and gender-matched patients who refused ECPT. Inclusion criteria were men and women aged 40–90 years, with symptomatic CAD documented by previous angiography (>70% stenosis in any coronary artery) and Canadian Cardiovascular Society (CCS) angina class II–IV. Exclusion criteria were unstable angina, acute myocardial infarction during the previous 3 months, aortic regurgitation, systemic hypertension >180/110 mm Hg, atrial fibrillation or ventricular premature beats that would interfere with ECPT triggering, clinically evident peripheral vascular disease, deep vein thrombosis, phlebitis and hemorrhagic diathesis, use of anticoagulants, pregnancy, abdominal aortic aneurysm, or refusal to sign informed consent. All medications, diet and interventions that could affect EPC number, such as cardiac rehabilitation and exercise, were held constant over the 10-week period following inclusion into the study.

The study was approved by the Institutional Ethics Committee of the Sheba Medical Center and registered in a public trial registry (NCT 00272571). All patients provided written informed consent.

The ECPT System

The ECPT system has been described previously [9]. In brief, the ECPT device (CardioAssist System; Cardiomedics Inc., Irvine, Calif., USA) uses the serial inflation of 3 sets of cuffs that wrap around the calves, thighs and buttocks. Inflation and deflation are timed to the patient's electrocardiogram, and the arterial pressure waveform is monitored noninvasively during 1 h. Overall duration for each ECPT course was 35 h extended over a 7-week period (1 h per day, 5 days a week).

Assessment of EPC Number and Function

EPC number and function were assessed by a flow-activated cell sorter (FACS) and an EPC colony-forming unit (CFU) assay, as previously described [13, 16]. Blood samples (30 ml) were drawn, after an overnight fast, from the antecubital vein, 1 week before and 1 week after a full 7-week course of ECPT, or after a 9-week follow-up period in the control group (the time interval between taking blood in both groups was 9 weeks). Samples were processed within 4 h after collection, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. For FACS analysis, recovered cells were washed and a volume of 70 μl (about 7×10^5) PBMCs was incubated for 25 min at room temperature with manufacturer-recommended concentrations of FITC-labeled monoclonal antibodies (mAbs) against human CD34 (Becton Dickinson), with phycoerythrin-conjugated mAbs against KDR (Becton Dickinson) and with PC5-conjugated mAbs against CD45 (Becton Dickinson). Isotype-identical antibodies served as controls (Becton Dickinson). After incubation, cells were lysed and washed before analysis. Each analysis included about 200,000 events using Epics XL (Beckman Coulter). The number of progenitor cells was expressed as the number of CD34+/KDR+ cells per 10^5 PBMCs.

For CFU assay, EPCs were cultured according to previously described techniques [13]. Briefly, EPC-CFU were assayed after 2 plantings and a 7-day culture on fibronectin-coated 24-well plates. Colonies were counted manually in a minimum of 3 wells by 2 observers who were unaware of the patients' clinical characteristics. Results were expressed as CFU/well in 4 separate wells from the same patient.

Confirmation of endothelial cell lineage was performed in samples from 5 subjects as previously described [13, 17]. Briefly, indirect immunostaining was performed with the use of endothelial-specific antibodies directed against vascular endothelial growth factor (VEGF) receptor 2 (KDR), CD31, von Willebrand factor and BS-1 lectin.

Assessment of Endothelium-Dependent and Endothelium-Independent Function

Endothelial function in the form of brachial artery flow-mediated dilatation (FMD) was assessed as previously described [9]. Briefly, imaging of the brachial artery proximal to the antecubital fossa was performed with the use of high-resolution ultrasonography (15–6 MHz linear array ultrasound model HP SON09-09OS 5500 CV system; Agilent Technologies Inc., Andover, Mass., USA). Endothelium-dependent FMD was assessed by using a pneumatic tourniquet placed around the forearm proximal to the target artery. The maximum vessel diameter after reactive hyperemia was measured relative to the baseline diameter. Endothelium-independent function was assessed after a nitroglycerin tablet (Nitrostat 0.4 mg; Parke-Davis, Morris Plains, N.J., USA) had been administered sublingually. The intra- and interobserver reproducibility for repeated measurements was 95 and 94%, respectively.

Other Laboratory Measurements

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of endothelial nitric oxide synthase which was shown to be an inhibitor of mobilization, differentiation and function of EPCs [18]. ADMA levels in plasma were measured by a competitive linked immunoassay (ADMA ELISA Kit; Immunodiagnostik

AG, Bensheim, Germany). The sensitivity of the test as reported by the manufacturer is 0.05 μmol/l.

Human VEGF levels were quantified in all patients' sera using ELISA according to the manufacturer's protocol (Quantikine hVEGF ELISA Kit; R&D Systems, Minneapolis, Minn., USA). The minimum level of detection was 5.0 pg/ml. The assay for C-reactive protein (CRP) was conducted according to the manufacturer's instructions (Dade Behring Inc.).

Statistical Analysis

Variables were expressed as means ± SD if normally distributed or as medians with interquartile ranges in parentheses if not. Differences between baseline clinical characteristics were evaluated by the χ^2 test for nominal variables and the Mann-Whitney U test for continuous variables. Because each patient in both groups was used as his or her own control, changes between baseline and 9 weeks in the control and treated groups were assessed with the Wilcoxon matched-pairs signed-ranks test. Univariate correlations were performed using Spearman's correlation coefficient. All tests were two-tailed. Differences of $p < 0.05$ were considered significant.

Results

Patient Characteristics

There were no significant differences in baseline characteristics between the ECPT (n = 15) and the control (n = 10) groups (table 1). All patients had angina with significant CAD (>70% stenosis confirmed by angiography). The percentage of patients with three-vessel disease in the ECPT and control groups was 73 and 70%, respectively.

ECPT Was Associated with Improved Number and Colony-Forming Capacity of EPCs

Changes in circulating EPCs were examined by 2 methods: the number of CD34+KDR+ EPCs in the circulation was quantified by FACS analysis and in vitro by counting EPC-CFUs (fig. 1).

ECPT increased the median number of circulating CD34+KDR+ EPCs from 10.2 (4.0–18.6) to 17.8 (7.3–28.3) per 10^5 mononuclear cells (p = 0.049). Correspondingly, the number of EPC-CFUs per well increased significantly after ECPT from 3.5 (2.0–6.1) before to 11.0 (6.2–14.3) after treatment (p = 0.010). In contrast, there were no significant differences in the number of CD34+KDR+ cells or EPC-CFUs in the control group before and after a 9-week follow-up: CD34+KDR+ cells: 10.0 (3.0–18.2) vs. 14.0 (4.0–22.8) cells/10^5 mononuclear cells, p = 0.430; EPC-CFUs: 6.9 (2.4–19.3) vs. 8.2 (5.1–16.3), p = 0.557 (fig. 2). EPC characteristics were confirmed by positive immunostaining for EPC markers including von Willebrand factor and BS-1 lectin (fig. 1).

Table 1. Patient baseline characteristics

	ECPT n = 15)	Control (n = 10)
Age, years[1]	69.8 ± 11.2	69.3 ± 9.6
Male	12 (80)	10 (80)
CCS angina class IV	6 (40)	3 (30)
CCS angina class III	7 (46.6)	5 (50)
CCS angina class II	2 (13.3)	2 (20)
LVEF, %[1]	51.3 ± 10.8	49.3 ± 10.9
Three-vessel disease	11 (73.3)	7 (70)
Previous revascularization (PTCA and/or CABG)	14 (93.3)	10 (100)
Diabetes mellitus	4 (26.6)	2 (20)
Hypertension	13 (86.6)	7 (70)
Hyperlipidemia	14 (93.3)	9 (90)
Current smoker	1 (6.6)	0 (0)
History of MI	12 (80)	9 (90)
Aspirin	14 (93.3)	9 (90)
β-Blockers	12 (80)	7 (70)
Statins	13 (86.6)	10 (100)
Long-acting nitrates	9 (60)	5 (50)
Diuretics	6 (40)	4 (40)
ACEI/ARB	9 (60)	7 (70)
CRP levels[2]	2.0 (0.9–2.9)	2.0 (1.3–5.3)

Data are presented as number of patients and percentage in parentheses unless otherwise indicated. All differences between groups are not statistically significant. ACEI = Angiotensin-converting enzyme inhibitor; ARB = angiotensin receptor blocker; CABG = coronary artery bypass graft; LVEF = left ventricular ejection fraction; MI = myocardial infarction; PTCA = percutaneous transluminal coronary angioplasty.

[1] Means ± SD.

[2] Medians with interquartile ranges in parentheses.

ECPT Was Associated with Improved Endothelial Function and Symptoms

ECPT significantly improved brachial artery endothelial function assessed by percent change in FMD after cuff deflation from 7.4 (1.1–13.5%) to 12.2 (5.4–17.7%), $p < 0.001$ (fig. 2). ECPT improved anginal symptoms assessed by CCS angina class from 3.0 (3.0–4.0) to 2.0 (2.0–3.0), $p < 0.001$. In contrast, there were no significant differences in endothelial-dependent FMD and CCS angina class in the control group before and after a 9-week follow-up: endothelial-dependent FMD: 10.5 (4.5–13.9) vs. 8.4 (5.5–16.9), p = 0.695; CCS angina class: 3.0 (2.5–4.0) vs. 3.0 (2.0–3.5), p = 0.500.

There were no significant differences in endothelium-independent function, assessed after nitroglycerin administration, in the ECPT group before and after treat-

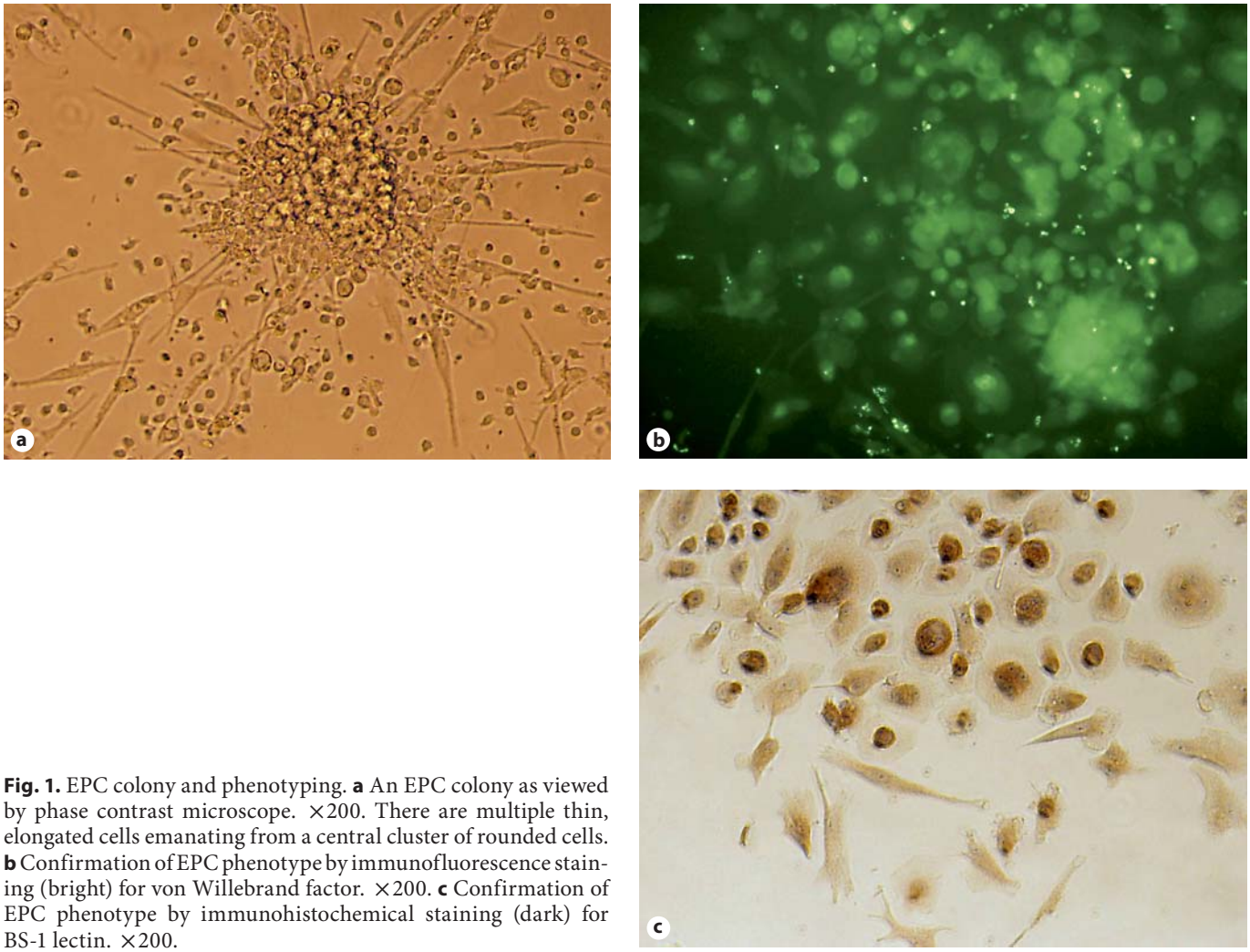

Fig. 1. EPC colony and phenotyping. **a** An EPC colony as viewed by phase contrast microscope. ×200. There are multiple thin, elongated cells emanating from a central cluster of rounded cells. **b** Confirmation of EPC phenotype by immunofluorescence staining (bright) for von Willebrand factor. ×200. **c** Confirmation of EPC phenotype by immunohistochemical staining (dark) for BS-1 lectin. ×200.

ment [13.0 (9.6–17.2) vs. 14.4 (9.2–21.0), p = 0.414] and in the control group before and after a 9-week follow-up [11.4 (6.3–21.2) vs. 17.7 (8.6–20.2), p = 0.652].

We also assessed the relationship between EPC-CFU counts and endothelial function by percent change in FMD which reflects endothelial function. As shown in figure 3, there was a significant correlation between the colony count and FMD (r = 0.46, p = 0.027). The correlation between EPC-CFUs and improvement in angina class did not reach statistical significance (r = –0.31, p = 0.115).

ECPT Was Associated with Reduced ADMA Levels in Plasma but Not VEGF or CRP

ECPT treatment was associated with a significant decrease in ADMA levels, an endogenous inhibitor of endothelial nitric oxide synthase and a marker of endothelial dysfunction, from 0.70 μmol/l (0.59–0.82) before to 0.60 μmol/l (0.49–0.73) after treatment (p = 0.006). On the other hand, there were no significant changes in ADMA levels in the control group before and after a 9-week follow-up: 0.63 (0.53–0.70) vs. 0.61 μmol/l (0.51–0.71), p = 0.322. ECPT did not affect VEGF or CRP levels either before or after treatment, VEGF levels changed from 64.9 (44.6–95.4) to 59.7 pg/ml (40.7–92.3), p = 0.330, and CRP levels from 2.0 (0.9–2.9) to 1.4 mg/l (0.8–3.8), p = 0.934. In the control group, there were no significant changes in VEGF or CRP levels before and after a 9-week follow-up: VEGF levels: 54.4 (51.9–60.8) vs. 44.7 pg/ml (35.6–48.9), p = 0.232; CRP levels: 2.0 (1.3–5.3) vs. 1.6 mg/l (1.1–5.6), p = 1.0.

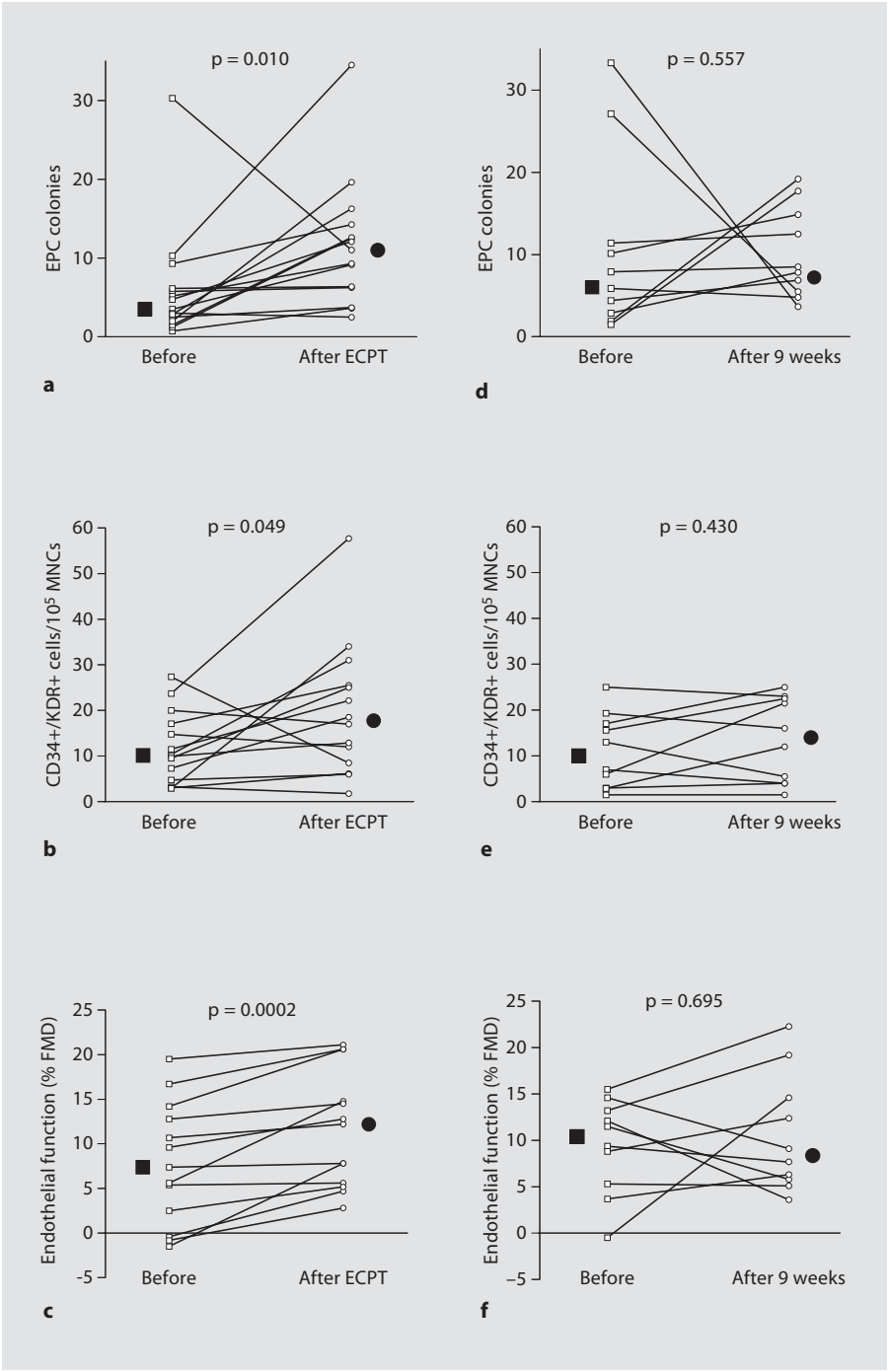

Fig. 2. ECPT increases the number and function of EPCs and improves endothelial function. The effects of ECPT before (squares) and after (circles) 9 weeks of treatment on the median number of EPC-CFUs assessed by colony assay (**a**), the median number of CD34+/KDR+ cells by flow cytometry (**b**) and brachial artery endothelial function assessed by median change in FMD (**c**) are shown. There was no significant change in the control group, at baseline (squares) and after 9 weeks (circles) for EPC-CFUs (**d**), CD34+/KDR+ cells (**e**) and FMD (**f**).

Discussion

The major new finding of the present study is that in patients with angina pectoris, short-term ECPT is associated with increased number and function of circulating EPCs. The improvement in EPC number and function was associated with improved brachial artery endothelial function and anginal symptoms. Our findings support previous reports [8, 9], suggesting that ECPT can be considered a viable treatment option in patients with refractory angina.

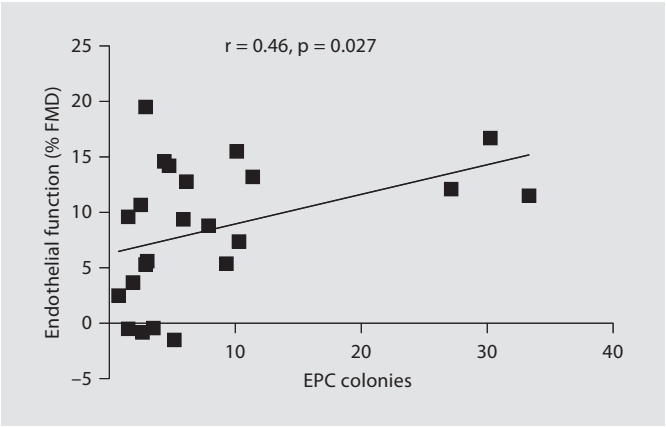

Fig. 3. The number of EPC-CFUs is correlated with brachial artery endothelial function assessed by percent change in FMD. The graph shows the values for all patients at baseline.

EPCs and Vascular Function

EPCs reside in bone marrow, from where they mobilize in response to neurohormonal stimuli such as trauma, stress or acute ischemia [19]. These cells have properties of embryonal angioblasts and can contribute to the maintenance of the vasculature and to the remodeling that accompanies new vessel growth [17, 20]. Circulating EPCs are depleted and exhausted in the presence of atherosclerosis risk factors [11, 12]. The number of circulating EPCs has been suggested to mirror vascular health and represent a marker by which to assess cardiovascular disease risk and prognosis [21, 22]. Lambiase et al. [23] showed that poor coronary collateral development is associated with reduced numbers of circulating EPCs. These observations prompted the concept that circulating EPCs may provide an endogenous repair tool to maintain, restore, replace and protect endothelial cells, and can predict disease progression and future cardiovascular events [11]. Furthermore, recent studies have provided increasing evidence that injection of EPCs after ischemia improves tissue neovascularization and possibly regeneration in animal models and in human patients [15].

In the present study, we assess EPC number by FACS and EPC function by CFU numbers, that have been correlated with EPC functional properties [24]. Although the definition of EPCs may vary from report to report [25], our definition has been accepted by many others [13, 21, 24]. In the current study, the changes or absence of changes in EPC and CFU number correlated with clinical measurements such as improved angina and brachial artery endothelial function.

Possible Mechanisms of ECPT

Little is known about the mechanisms by which ECPT improves endothelial function and new vessel formation [1]. Suggested mechanisms contributing to the clinical benefit of ECPT include improvement in endothelial function, promotion of coronary collateralization, enhancement of ventricular function, peripheral effects similar to those observed with regular physical exercise, and nonspecific placebo effects [1]. ECPT has been demonstrated to increase plasma nitric oxide and decrease plasma endothelin-1 levels, providing some insight into how ECPT may improve endothelial function [26]. ECPT can stimulate angiogenesis and collateral formation as demonstrated in a dog model of ischemia [10]. The present study proposes a new mechanism of EPC stimulation. Our observation may bridge the gap between studies showing the beneficial effects of ECPT on endothelial function and those showing the contribution of EPCs to the maintenance and remodeling of the vasculature in CAD patients.

Interestingly, in the present study, ECPT was associated with decreased levels of ADMA, with no change in VEGF or CRP levels. ADMA is a methylated amino acid produced by the endothelium and is able to outcompete L-arginine as a substrate for endothelial nitric oxide synthase, leading to endothelial dysfunction. ADMA has been demonstrated to inhibit EPC mobilization, differentiation and function [18]. In the present study, we also examined the levels of VEGF (previously thought to influence EPC levels) [14], and the levels of CRP which were shown to have proapoptotic effects on EPCs, cause EPC dysfunction and inhibit EPC migration [27]. We found no significant change in CRP or VEGF levels between the groups. Possible explanations for the lack of correlation between EPC and VEGF levels may include: other mediators besides VEGF that regulate EPC recruitment, a time lag between the increase in VEGF levels and the rise in circulating EPCs, and a possible regulation of VEGF receptors on EPCs without a significant change in VEGF levels.

Finally, it is possible that by increasing endothelial shear stress, ECPT regulates other potential chemokines or growth factors which stimulate mobilization of EPCs from bone marrow and increase the number of circulating EPCs and their functional capacity [28].

Summary and Further Research

The present study suggests that ECPT may be associated with an increase in the number and colony-forming capacity of circulating EPCs. However, due to its small,

unblinded, nonrandomized nature, the results of our study should be treated with caution. In order to confirm our findings and support the potential role of ECPT as a noninvasive method for EPC stimulation and vascular health, a randomized controlled trial is warranted in a large group of patients with refractory angina pectoris. The time course and durability of the EPC response to ECPT could also be a valuable area of further research.

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