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Increase in Circulating Endothelial Progenitor Cells by Statin Therapy in Patients With Stable Coronary Artery Disease

Mariuca Vasa, MD; Stephan Fichtlscherer, MD; Klaudia Adler; Alexandra Aicher, MD; Hans Martin, MD; Andreas M. Zeiher, MD; Stefanie Dimmeler, PhD

Background—Therapeutic neovascularization may constitute an important strategy to salvage tissue from critical ischemia. Circulating bone marrow–derived endothelial progenitor cells (EPCs) were shown to augment the neovascularization of ischemic tissue. In addition to lipid-lowering activity, hydroxymethyl glutaryl coenzyme A reductase inhibitors (statins) reportedly promote the neovascularization of ischemic tissue in normocholesterolemic animals.

Methods and Results—Fifteen patients with angiographically documented stable coronary artery disease (CAD) were prospectively treated with 40 mg of atorvastatin per day for 4 weeks. Before and weekly after the initiation of statin therapy, EPCs were isolated from peripheral blood and counted. In addition, the number of hematopoietic precursor cells positive for CD34, CD133, and CD34/kinase insert domain receptor was analyzed. Statin treatment of patients with stable CAD was associated with an \approx 1.5-fold increase in the number of circulating EPCs by 1 week after initiation of treatment; this was followed by sustained increased levels to \approx 3-fold throughout the 4-week study period. Moreover, the number of CD34/kinase insert domain receptor–positive hematopoietic progenitor cells was significantly augmented after 4 weeks of therapy. Atorvastatin treatment increased the further functional activity of EPCs, as assessed by their migratory capacity.

Conclusion—The results of the present study define a novel mechanism of action of statin treatment in patients with stable CAD: the augmentation of circulating EPCs with enhanced functional activity. Given the well-established role of EPCs of participating in repair after ischemic injury, stimulation of EPCs by statins may contribute to the clinical benefit of statin therapy in patients with CAD. (*Circulation*. 2001;103:2885-2890.)

Key Words: coronary disease ■ angiogenesis ■ endothelium

Blood cholesterol lowering with statins is well established as a long-term strategy to reduce death and ischemic cardiovascular events in patients with stable coronary artery disease (CAD).¹⁻³ Major mechanisms by which lipid lowering is thought to improve outcome include preventing the development of new atherosclerotic lesions and stabilizing existing atherosclerotic plaques.⁴ In addition, statins can reduce vascular inflammation,⁵ decrease platelet aggregability and thrombus deposition,⁶ and increase endothelium-derived nitric oxide production.⁷ Most recently, statins have been reported to promote the neovascularization of ischemic tissue in normocholesterolemic animals.⁸

Therapeutic neovascularization may constitute an important way to salvage tissue from critical ischemia.⁹ Neovascularization in the adult is thought to result exclusively from the migration and proliferation of preexisting, fully differentiated

endothelial cells (a process referred to as angiogenesis).¹⁰ Recent studies, however, demonstrated that circulating bone marrow–derived endothelial progenitor cells (EPCs) home to sites of neovascularization and differentiate into endothelial cells in situ^{11,12} in a manner consistent with a process termed vasculogenesis.¹³ Importantly, mobilization of bone marrow–derived EPCs augments the neovascularization of ischemic tissue,¹⁴ thus suggesting that the mobilization of EPCs might represent a useful strategy for clinical therapy of ischemic heart disease.

Therefore, we tested the hypothesis that statin therapy might augment circulating EPCs in patients with stable CAD.

Methods

Characteristics of Study Patients and Healthy Controls

Fifteen patients with angiographically documented CAD were prospectively studied. The patient characteristics are summarized in

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TABLE 1. Baseline Clinical Characteristics of the Patients

Age, y	64.3±2.6
Men, n (%)	12 (80)
No of diseased coronary arteries, n (%)	
1	9 (60)
2	2 (13.3)
3	4 (26.7)
Medical history, n (%)	
Hypertension	11 (73.3)
Diabetes mellitus	5 (33.3)
Current smoking	3 (20)
Family history of CAD	3 (20)
Left ventricular ejection fraction	58.3±2.2
Current medication, n (%)	
Aspirin	11 (73.3)
Cumarines	3 (20)
β-Blocker	12 (80)
ACE inhibitors	11 (73.3)
Insulin	3 (20)
Lipid profile, mg/dL	
Total cholesterol	213±12.2
LDL cholesterol	145±11.5
HDL cholesterol	43.5±2.3
Triglycerides	131±17.4

Data are expressed as mean±SE or n (%). n=15.

Table 1. Patients with concomitant inflammatory or malignant disease were excluded and, to avoid any potentially confounding effect of myocardial ischemia on EPC kinetics, none of the patients had flow-limiting coronary artery stenosis (>50% diameter reduction) at the time of inclusion into the study. In addition, patients with unstable angina or myocardial infarction within the preceding 3 months were excluded. None of the patients had previously been treated with a statin. The LDL cholesterol serum levels ranged from 57 to 207 mg/dL at the time of inclusion into the study.

The age-matched healthy control group (n=9) consisted of 3 women and 6 men with a mean age of 60±5.3 years without any evidence of CAD by history and physical examination. In an additional 5 healthy volunteers (mean age 36±6.3 years), EPC kinetics were investigated 3 times over a 4-week period to assess any potential spontaneously occurring changes in EPC numbers.

Study Protocol

The 15 study patients received 40 mg of atorvastatin (Pfizer) per day over 4 weeks. Before and weekly after the initiation of statin therapy, 40 mL of venous blood was collected to measure circulating EPCs, serum cholesterol levels, and vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α), and granulocyte macrophage colony-stimulating factor (GM-CSF) serum levels. Informed consent was obtained from all patients and healthy volunteers, and the study protocol was approved by the local Ethics Committee of the University of Frankfurt.

EPC Culture Assay

Mononuclear cells were isolated by density-gradient centrifugation with Biocoll from 20 mL of peripheral blood. Immediately after isolation, 4×10⁶ mononuclear cells were plated on 24-well culture dishes coated with human fibronectin and gelatin (Sigma) and maintained in endothelial basal medium (EBM, CellSystems) supplemented with EGM SingleQuots and 20% FCS. After 4 days in

culture, nonadherent cells were removed by a thorough washing with PBS, and adherent cells underwent cytochemical analysis.

Characterization of EPCs

To detect the uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (DiLDL), cells were incubated with DiLDL (2.4 μg/mL) at 37°C for 1 hour. Cells were then fixed with 2% paraformaldehyde for 10 minutes, and lectin staining was performed by incubation with fluorescein isothiocyanate (FITC)-labeled Ulex europaeus agglutinin I (lectin, 10 μg/mL; Sigma) for 1 hour. After the staining, samples were viewed with an inverted fluorescent microscope (Zeiss). Dual-stained cells positive for both lectin and DiLDL were judged to be EPCs, and they were counted per well. Two to three independent investigators evaluated the number of EPCs per well by counting 3 randomly selected high-power fields.¹⁵

To detect the expression of endothelial marker proteins, EPCs were detached with 1 mmol/L EDTA in PBS, followed by repeated gentle flushing through a pipette tip. Cells were incubated for 15 minutes with phycoerythrin-labeled monoclonal antibodies against human kinase insert domain receptor (KDR) (Sigma) and human vascular endothelium-cadherin and a FITC-labeled monoclonal antibody against von Willebrand factor. After treatment, the cells were lysed and fixed in 4% paraformaldehyde. CD14-positive monocytes were obtained by positive selection with CD14 immunomagnetic microbeads (Milteny, Biotec) using an auto-magnetic cell sorting cell separation device. Single and 2-color flow cytometric analysis were performed using a fluorescence-activated cell sorter (FACS) SCAN flow cytometer (Becton Dickinson).

Flow Cytometry Analysis

A volume of 100 μL of peripheral blood was incubated for 15 minutes in the dark with monoclonal antibodies against human KDR (Sigma), the FITC-labeled monoclonal antibody against human CD45 (Becton Dickinson), the phycoerythrin-conjugated monoclonal antibody against human CD133 (Milteny), and the FITC- or phycoerythrin-conjugated monoclonal antibody against human CD34 (Becton Dickinson). Isotype-identical antibodies served as controls (IgG1-phycoerythrin and IgG2a-FITC, Becton Dickinson). Each analysis included 60 000 events.

Migration Assay

Isolated EPCs were detached using 1 mmol/L EDTA in PBS (pH 7.4), harvested by centrifugation, resuspended in 500 μL of EBM, counted, and placed in the upper chamber of a modified Boyden chamber. The chamber was placed in a 24-well culture dish containing EBM and human recombinant VEGF (50 ng/mL). After 24 hours of incubation at 37°C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cell nuclei were stained with 4',6-diamidino-phenylidole. Migrating cells into the lower chamber were counted manually in 3 random microscopic fields.¹⁶

Serum VEGF, GM-CSF, and TNF levels

Serum levels of the cytokines were measured by a high-sensitive ELISA assay (R&D Systems) according to the manufacturer's instructions. Samples were checked by serial dilution, and measurements were performed at least in duplicate.

Statistical Analysis

Data are expressed as mean±SEM. Continuous variables were tested for normal distribution with the Kolmogorov-Smirnov test and compared by 1-way ANOVA. Categorical variables were compared using the χ² test and the Fisher exact test. In the case of non-normal distribution, nonparametric tests were used (Mann-Whitney U test or Kruskal-Wallis ANOVA on ranks). Differences in EPC number and FACS parameters were examined by repeated-measures ANOVA. Linear regression analysis and nonparametric bivariate correlation (Spearman rank correlation coefficient) were used to compare increases in EPCs versus a reduction of LDL cholesterol levels.

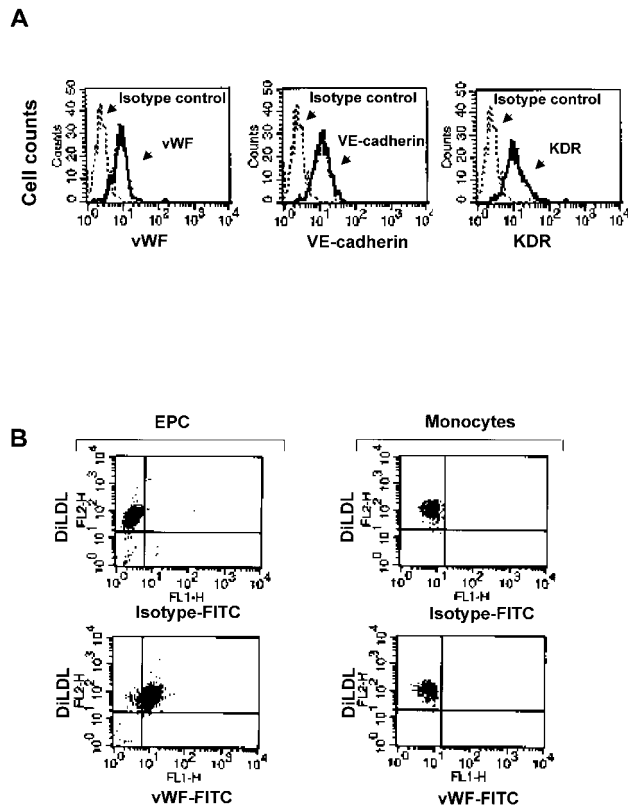


Figure 1. EPC characteristics. A, The expression of KDR receptor, von Willebrand factor (vWF), and vascular endothelium (VE)-cadherin in EPCs was analyzed by FACS and compared with isotype controls. B, EPCs (left) were compared with CD14-positive isolated monocytes (right). DiLDL uptake and von Willebrand factor staining were determined by FACS. Quadrants were set on the basis of FITC isotype controls and cells without DiLDL incubation. Representative images from 3 to 20 experiments are shown.

Statistical significance was assumed if a null hypothesis could be rejected at $P < 0.05$. All statistical analyses were performed with SPSS for Windows 7.0.

Results

Effect of Atorvastatin on EPCs

EPCs were isolated and cultivated from peripheral blood and characterized as dual-stained cells positive for DiLDL and lectin. In addition, the endothelial phenotype was confirmed by demonstrating the expression of the endothelial marker proteins KDR, vascular endothelium-cadherin and von Willebrand factor by flow cytometry (Figure 1A). Moreover, EPCs were double-positive for DiLDL uptake and von Willebrand factor expression (Figure 1B). To exclude the possibility that these cells could be monocytes, the same staining procedure was repeated with isolated CD14-positive monocytes. As expected, monocytes were positive for DiLDL uptake but negative for von Willebrand factor expression (Figure 1B).

Before initiating statin therapy, the number of EPCs was lower but not significantly reduced in patients with CAD (190 ± 49 EPCs/mm²) compared with age-matched healthy controls (310 ± 55 EPCs/mm², $P = 0.052$). Treatment with 40 mg of atorvastatin per day was associated with a significant

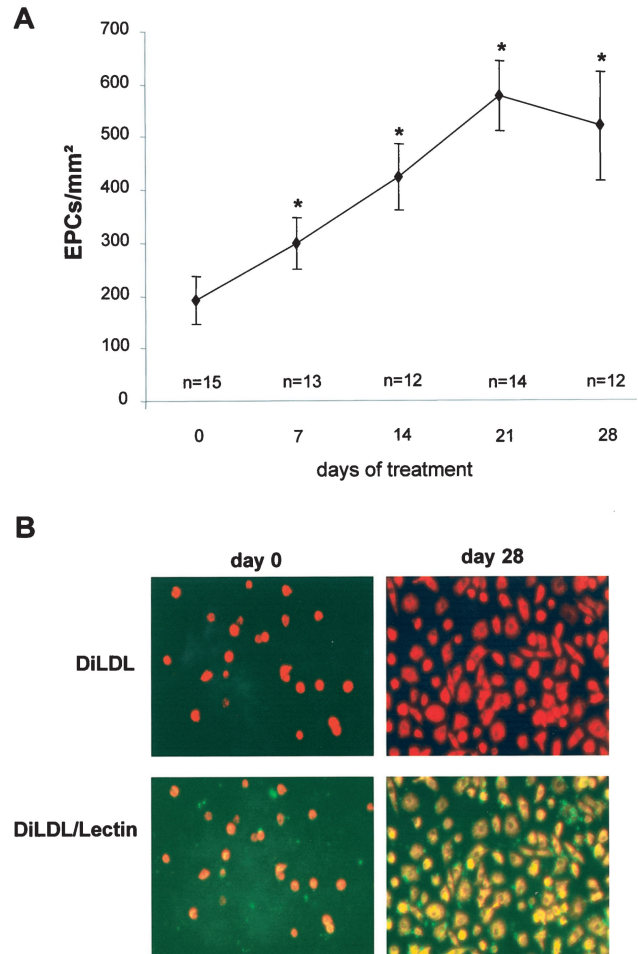


Figure 2. A, Atorvastatin therapy augments EPCs. EPCs were isolated before and after patients with stable CAD were treated with atorvastatin (40 mg/d). EPCs were characterized as adherent cells with double-positive staining for DiLDL and lectin. $*P < 0.05$ vs day 0. Representative images are shown in B.

increase in the number of circulating EPC in patients with CAD (Figure 2, $P < 0.05$ for trend). As illustrated in Figure 2A, a significant ($P = 0.016$), ≈ 1.5 -fold increase in EPCs was observed after only 1 week of treatment; this was followed by a further increase to 3-fold at week 2 and was sustained at >4 -fold throughout the 4-week study period. In addition, atorvastatin treatment also augmented EPC numbers in 3 healthy volunteers (3 men aged 50 ± 11 years) from 318 ± 68 to 494 ± 68 and 677 ± 101 EPCs/mm² after 1 and 3 weeks, respectively ($P < 0.05$). In contrast, repeated measurements of circulating EPCs in 5 healthy control subjects without statin treatment over a 4-week period revealed essentially identical values (331 ± 46 , 305 ± 29 , and 287 ± 50 EPCs/mm² at baseline, 2 weeks, and 4 weeks, respectively). Thus, statin treatment significantly augments the number of circulating EPC within 1 week of treatment.

Effect of Atorvastatin on Hematopoietic Progenitor Cells

EPCs are thought to derive from CD34-positive hematopoietic progenitor cells.^{11,17-19} The subset of endothelial precursor cells is characterized by the coexpression of endothelial

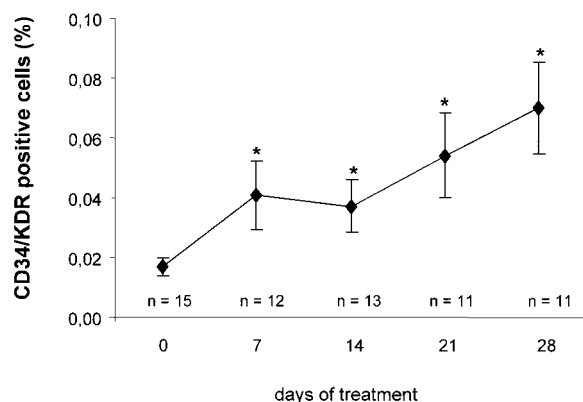


Figure 3. Atorvastatin therapy increases CD34/KDR-positive hematopoietic stem cells. CD34/KDR-positive lymphocytes were analyzed in peripheral blood from patients with stable CAD before and weekly after atorvastatin therapy, as indicated. * $P < 0.05$ vs day 0. Data are mean \pm SEM.

marker proteins such as VEGF receptor 2 (KDR).^{17–19} The baseline number of circulating CD34/KDR-positive cells was reduced in patients with CAD compared with healthy age-matched volunteers ($0.0173 \pm 0.004\%$ versus $0.029 \pm 0.006\%$ in healthy controls, $P = 0.116$).

Treatment with atorvastatin led to an increase in CD34/KDR-positive cells starting 7 days after initiating treatment (Figure 3). In contrast, the overall number of circulating CD34-positive cells did not change during the treatment period ($0.067 \pm 0.01\%$ at baseline versus $0.076 \pm 0.013\%$ after 4 weeks, $P = \text{NS}$). Likewise, the number of CD133-positive hematopoietic progenitor cells, which represent a more immature subset of CD34-positive cells, remained unchanged ($0.07 \pm 0.012\%$ versus $0.05 \pm 0.009\%$ after 4 weeks, $P = \text{NS}$). Finally, atorvastatin treatment did not affect the total number of mononuclear cells ($0.82 \pm 0.08 \times 10^6$ cells/mL versus $0.85 \pm 0.09 \times 10^6$ cells/mL after 4 weeks, $P = \text{NS}$). Again, in healthy control subjects without statin treatment, all parameters tested remained constant during the observation period, whereas statin treatment in 3 healthy volunteers significantly increased the number of CD34/KDR-positive cells to 365% after 3 weeks of treatment ($P = 0.028$). Thus, a 4-week period of statin treatment seems to stimulate the differentiation of CD34-positive cells into EPCs rather than to augment the numbers of circulating hematopoietic progenitor cells.

Effects of Atorvastatin on the Migratory Capacity of Isolated EPCs

To assess the potential functional effects of statin therapy on EPCs, we analyzed the migratory capacity of isolated EPCs in response to VEGF in a subset of 12 patients before and after 3 and 4 weeks of treatment with 40 mg of atorvastatin per day. At baseline, patients with stable CAD had lower numbers of migrating EPCs than healthy volunteers (11 ± 5.8 versus 31.9 ± 4.8 migrating EPCs per high-power field, respectively, $P < 0.05$). As illustrated in Figure 4, atorvastatin treatment significantly augmented the migration of isolated EPCs from 11.5 ± 5.9 to 34.6 ± 13.5 migrating EPCs/high power field after 3 weeks ($P = 0.009$). Thus, statin therapy

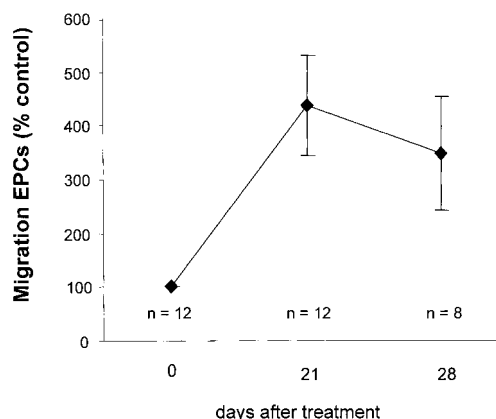


Figure 4. Migratory capacity of EPCs after statin therapy. Migration of EPCs was analyzed in a subset of 12 patients by using a modified Boyden chamber, as outlined in Methods. Data are mean \pm SEM.

increases the number of circulating EPCs and stimulates the functional activity of these cells.

Effects of Atorvastatin on Serum Cholesterol and Cytokine Levels

Treatment with 40 mg of atorvastatin per day resulted in a decrease in LDL serum cholesterol levels (Figure 5). However, neither the absolute number of EPCs at baseline nor the EPC kinetics during treatment correlated with LDL cholesterol levels ($r = 0.377$, $P = 0.165$) or statin-induced changes in LDL cholesterol serum levels ($r = -0.017$, $P = 0.955$; $r = 0.134$, $P = 0.694$; and $r = -0.199$, $P = 0.496$ at 1, 2, and 3 weeks, respectively). In addition, statin treatment did not affect serum levels of VEGF, GM-CSF, or TNF- α (Table 2), which all modulate EPC mobilization or angiogenesis in vivo.^{14,20,21}

Discussion

The results of the present study demonstrate that statin therapy is associated with an increase in the number of circulating EPCs in patients with stable CAD. The increase in

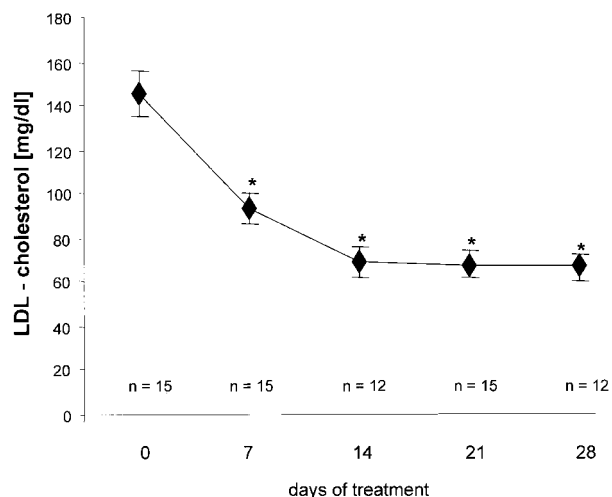


Figure 5. Effects of statin therapy on LDL cholesterol serum levels. LDL cholesterol levels were measured before and after atorvastatin therapy. * $P < 0.05$ vs day 0.

TABLE 2. Initial and Follow-Up Serum Cytokine Levels in Patients With Stable CAD Undergoing Atorvastatin Treatment

Variable	Day 0	Day 14	Day 28
VEGF, pg/mL	67.7±16.7	38.9±7.3	63.7±16.1
GM-CSF, pg/mL	0.23±0.05	0.26±0.05	0.17±0.05
TNF- α , pg/mL	1.7±0.3	1.7±0.3	1.5±0.2

Data are expressed as mean±SE.

EPCs was statistically significant as early as 1 week after the initiation of atorvastatin treatment, and it plateaued at a 3-fold increase at 3 to 4 weeks of therapy. The increased number of EPCs was paralleled by an enhancement of the migratory capacity of isolated EPCs. Mobilization of circulating EPCs with enhanced functional activity might contribute to the well-established beneficial effects of statins in patients with CAD.

Although the proportional contribution of angiogenesis and vasculogenesis to the neovascularization of adult tissue remains to be determined, it is well established that EPCs participate in repair after ischemic injury. Experimental hindlimb ischemia in mice increases the number of circulating EPCs by >400%.¹⁴ The angiogenic growth factor VEGF, which is upregulated in the ischemic myocardium of patients with myocardial infarction,²² has been shown to mobilize EPCs in both mice and men.^{16,20} Finally and most importantly, transplantation of blood-derived EPCs significantly augmented ischemia-induced neovascularization of the hindlimb^{23,24} and promoted limb salvage in nude mice.¹⁵ Thus, the finding that statin therapy augments the number of circulating EPCs in patients with CAD implies that vasculogenesis may contribute to statin-mediated repair after ischemic injury, which was very recently demonstrated for the rabbit model of hindlimb ischemia.⁸

Upregulation of angiogenic growth factors constitutes a fundamental survival response to tissue ischemia. Therefore, VEGF seems to be a key regulatory cytokine orchestrating endogenous neovascularization by modulating endothelial cell migration and proliferation and circulating cellular elements.⁹ Recent experimental and clinical studies have demonstrated that VEGF affects endothelial cell migration and proliferation and significantly alters the kinetics of EPCs.^{11,20} Treating mice with recombinant human VEGF₁₆₅ increases the number of EPCs by 245% and 214% after 1 and 4 days, respectively.¹⁶ Likewise, in patients with critical limb ischemia receiving VEGF gene transfer, the number of EPCs increased by 154% and 153% at days 14 and 28 after treatment, respectively, in parallel with an \approx 2-fold increase in VEGF plasma levels.²⁰ In the present study using a culture assay identical to one used previously to quantify circulating EPC kinetics, a >3-fold increase in circulating EPCs was observed 2 weeks after initiating atorvastatin treatment in patients with stable CAD. Thus, the effects of statin therapy in augmenting circulating EPCs seem to be at least comparable to the effects of exogenous VEGF administration.

The mechanisms mediating the effects of statins on EPC kinetics in humans remain to be determined. Our data suggest that the modulation of EPC kinetics after statin treatment is unrelated to the decrease in serum LDL cholesterol levels. In

a manner similar to the mobilization of hematopoietic progenitor cells,²⁵ cytokines like GM-CSF have also been shown to exert potent stimulatory effects on EPC kinetics.¹⁴ However, in the present study, atorvastatin did not affect the serum levels of GM-CSF or TNF- α in patients with CAD. Likewise, VEGF serum levels did not significantly change during atorvastatin treatment. It is known that statins can regulate a variety of intracellular signaling pathways, including Rho GTPase, thereby stabilizing endothelial nitric oxide synthase (eNOS) mRNA levels.⁷ Moreover, statins were recently shown to stimulate the protein kinase Akt,⁸ which activates the enzymatic activity of eNOS,^{26,27} mediates VEGF-induced endothelial cell migration,^{28,29} and thereby plays an important role in mature endothelial cells.³⁰ Thus, one may speculate that statin-induced stimulation of the Akt/eNOS pathway might contribute to the observed effects of statins on the functional improvement of EPCs.

Obviously, because of the limitations imposed by studying patients, we cannot determine the molecular pathway(s) responsible for statin-induced augmentation in circulating EPCs in our patients with stable CAD. Moreover, because of the potent beneficial effect of statins in normocholesterolemic patients with CAD, a placebo control group cannot be provided for ethical reasons. However, the finding that statin therapy also increases EPC levels in healthy volunteers, whereas EPC levels remained unchanged in the untreated healthy control group, supports the hypothesis that statins directly affect EPC levels.

Interestingly, patients with CAD revealed reduced EPC numbers and migration. Although the data did not reach statistical significance with respect to EPC levels, one may speculate that individual risk factors contribute to the impairment of EPC numbers and function. Further studies with larger patient numbers are required to elucidate the potential contribution of specific risk factors for CAD on EPC number and function.

In conclusion, the results of the present study define a novel mechanism of action of statin treatment in patients with stable CAD: namely, the augmentation of circulating EPCs with enhanced functional activity. Our data further suggest that statin treatment seems to stimulate the differentiation of a subset of endothelial precursor cells into EPCs rather than augmenting the number of circulating hematopoietic stem cells. Given the well-established role of EPCs participating in repair after ischemic injury, the mobilization of EPCs by statins may contribute to the clinical benefit of statin therapy in patients with CAD, in addition to the effects of statins on serum cholesterol levels and atherosclerotic plaque stabilization. The potential of statins to improve the neovascularization of ischemic tissue suggests that statin therapy may support one of the most fundamental survival responses to maintain tissue viability in the face of acute or chronic myocardial ischemia in patients with obstructive CAD. In fact, statin therapy was recently shown to rapidly enhance coronary blood flow in patients with stable CAD³¹ and to reduce myocardial ischemia after an acute ischemic episode within a few weeks of treatment.³²

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