Statin Therapy Accelerates Reendothelialization
A Novel Effect Involving Mobilization and Incorporation of Bone Marrow–Derived Endothelial Progenitor Cells

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Background—Primary and secondary prevention trials suggest that statins possess favorable effects independent of cholesterol reduction. We investigated whether statin therapy may also accelerate reendothelialization after carotid balloon injury.

Methods and Results—Simvastatin treatment in 34 male Sprague-Dawley rats accelerated reendothelialization of the balloon-injured arterial segments (reendothelialized area at 2 weeks, 12.3\(\pm\)1.8 versus 5.4\(\pm\)1.1 mm\(^2\), \(P<0.01\)) and resulted in a dose-dependent (0.2 or 1 mg/kg IP) significant reduction in neointimal thickening at 2, 3, and 4 weeks compared with saline-injected controls (n=18). To elucidate the mechanism, we investigated the contribution of bone marrow–derived endothelial progenitor cells (EPCs) by bone marrow transplantation from Tie2/lacZ mice to background mice or nude rats. X-gal staining of mouse carotid artery specimens revealed a 2.9-fold increase in the number of \(\beta\)-gal–positive cells per square millimeter appearing on the carotid artery luminal surface at 2 weeks, and double-fluorescence immunohistochemistry disclosed a significant 5-fold increase in the number of double-positive cells (\(\beta\)-gal, isoelectric B4) on the luminal surface in carotid arteries of statin-treated nude rats (20\(\pm\)3 versus 4\(\pm\)1 cells/mm surface length, \(P<0.005\)). Statins increased circulating rat EPCs (2.4-fold at 2 weeks and 2.5-fold at 4 weeks, \(P<0.001\)) and induced adhesiveness of cultured human EPCs by upregulation of the integrin subunits \(\alpha_\beta\), \(\beta_\gamma\), \(\alpha_\delta\), and \(\beta_\delta\) of human EPCs as shown by reverse transcription–polymerase chain reaction and fluorescence-activated cell sorting.

Conclusions—These findings establish additional mechanisms by which statins may specifically preempt disordered vascular wall pathology and constitute physiological evidence that EPC mobilization represents a functionally relevant consequence of statin therapy. (Circulation. 2002;105:3017-3024.)

Key Words: coronary disease ■ endothelium ■ angiogenesis ■ statins ■ cells

The use of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors, or statins, constitutes a well-established and potent strategy for reducing cholesterol levels in human subjects. Moreover, primary and secondary prevention trials suggested, and laboratory investigations established, that statins possess favorable effects independent of cholesterol reduction. In particular, statins have been shown to decrease neointimal thickening in animal models of carotid injury and reduce clinical events and angiographic restenosis after coronary stent implantation.

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These findings have been attributed, at least in part, to inhibition of vascular smooth muscle cell proliferation. Several groups, however, have demonstrated profound, positive effects of statins on endothelial cell function. Most recently, in vivo studies have established that statins may promote angiogenesis in ischemic limbs, analogously to endothelial cell mitogens. These findings are of interest because drugs and cytokines known to favorably influence endothelial cell function and promote angiogenesis have also been shown to promote reendothelialization after vascular injury.

Reendothelialization at sites of spontaneous or iatrogenic disruption has classically been thought to result from the migration and proliferation of endothelial cells from viable endothelium adjacent to the site of injury. Neighboring endothelial cells, however, may not constitute the exclusive basis for endothelial repair. Circulating cells derived from the bone marrow and exhibiting phenotypic features of endothelial cells are capable of homing to sites of endothelial disruption and incorporating into nascent endothelium.

More recently, 2 groups have documented in animals and human subjects that statins may mobilize bone mar-
row-derived endothelial progenitor cells (EPCs). The extent to which this property of statins may contribute to biologically relevant activities, including angiogenesis and reendothelialization, however, has not been demonstrated in vivo. We therefore tested the hypothesis that statin therapy may accelerate reendothelialization after balloon denudation.

**Methods**

**Rat Model of Carotid Denudation**
Male Sprague-Dawley rats (n=52, Charles River, Wilmington, Mass; 8 months old, 550 to 600 g, retired breeders) under general anesthesia underwent carotid balloon denudation with a 2F Fogarty balloon catheter as previously described.¹⁴ The length of the injured segment was similarly defined proximally by the carotid bifurcation and distally by the edge of the omohyoid muscle. All procedures were performed in accordance with the St Elizabeth’s Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals.

**Statin Therapy**
Animals received daily intraperitoneal injections of simvastatin¹¹ activated by alkaline hydrolysis according to the manufacturer’s instructions in physiological doses (0.2 or 1 mg/kg, Merck) or saline 0.9% IP until they were killed (at 2, 3, or 4 weeks). Cholesterol levels were analyzed by 1% agarose gel electrophoresis with a 100-bp ladder (Life Technologies) and quantified with the UV imager (Eagle-Eye, Stratagene). RNA 18S internal standards were used (Ambion). RT-PCR products were analyzed by 1% agarose gel electrophoresis with a 100-bp ladder (Life Technologies) and quantified with the UV imager (Eagle-Eye, Stratagene).

**Cell Adhesion Assay**
After 3 days of incubation with simvastatin 1 µmol/L, human EPCs were detached with PBS and gently detached with 0.5 mmol/L EDTA in PBS. After centrifugation and resuspension in basal complete medium, 5% FCS, identical cell numbers were placed onto fibronectin-coated culture dishes and incubated for 30 minutes at 37°C. Adherent cells were counted by independent blinded investigators.

**Histochemical Analysis**
To measure the reendothelialized area, animals were perfused either in vivo with Evans blue dye¹⁴ (Sigma) or in vivo with BS1-lectin or isoelectin B4 staining to identify endothelium (Vector Laboratories) at predetermined time points immediately before death. For determination of intima/media (I/M) ratio, serial cross sections of paraffin-embedded specimens were stained with elastic trichrome stain.

**Semiquantitative Reverse Transcription–Polymerase Chain Reaction**
Expression of the surface receptor integrin subunits α₅, β₃, α₆, β₃, and vascular cell adhesion molecule (VCAM)-1 as well as CD31 was evaluated by reverse transcription–polymerase chain reaction (RT-PCR). RNA of cultured human EPCs or HUVECs was extracted by the Ambion RNAqueous kit. cDNA synthesis was performed by the Ambion RevertAid MMLV reverse transcriptase (Fermentas) and synthesized cDNA was amplified using primer pairs specific for each gene (Table I). Amplified products were separated by electrophoresis on a 1.5% agarose gel stained with ethidium bromide, and the intensity of the bands was determined by the ImageQuant software (Molecular Dynamics).

**Fluorescence-Activated Cell Sorting Analysis**
Fluorescence-activated cell sorting (FACS) was used to detect the expression of cell-surface integrins and endothelial lineage antigens on EPCs. FITC-conjugated antibodies for integrin subunits α₁ (CD49E), β₁ (CD29), anti-α₅β₃ (all Chemicon), and anti-CD68 were used.

**Human EPC Culture**
Peripheral blood mononuclear cells were isolated from the blood of human volunteers by density gradient centrifugation with Histopaque-1077 (Sigma) and cultured until day 7 on human fibronectin as previously described.

**Human Umbilical Vein Endothelial Cell Monolayer**
Three days after simvastatin exposure, human EPCs were detached with PBS (0.5 mmol/L EDTA) and labeled with the fluorescence marker DiI for cell tracking (Biomedical Technologies). Identical numbers of DiI-labeled EPCs were incubated for 24 hours on a human umbilical vein endothelial cell (HUCV) monolayer plated on fibronectin-coated 6-well culture dishes with or without pretreatment with tumor necrosis factor-α (1 ng/mL) for 12 hours. Nonadhesive cells were removed after 3 hours by washing with PBS. The total numbers of adhesive EPCs in each well were counted in a blinded manner.

**Human EPCs**
Peripheral blood mononuclear cells were isolated from the blood of Sprague-Dawley rats that had been treated with saline or simvastatin (1 mg·kg⁻¹·d⁻¹ IP) at 2 and 4 weeks after balloon denudation by density gradient centrifugation with Histopaque-1083 (Sigma). Four days after EPC culture on rat vitronectin + 0.5% gelatin, EPCs were assayed by costaining with acLDL/DiI (Biomedical Technologies) and FITC-conjugated BS-1 lectin (Vector). Fluorescence microscopy identified double-positive cells per square millimeter as EPCs, which were counted by investigators blinded to treatment.
and no-statin groups were similar (18.7 versus 17.5 mm²). Statin treatment produced dose-dependent accelerated reendothelialization of the balloon-injured arterial segments (Figure 1A). At 2 weeks, the reendothelialized area of statin-treated rats (0.2 mg/kg) was 12.3±1.8 mm², or 66.6±9.9% of the total denuded area; in contrast, the reendothelialized area in the no-statin group measured 5.4±1.1 mm², or 31.6±5.9% of the denuded area (P<0.01). At 4 weeks, reendothelialization was nearly complete in statin-treated animals (17.9±0.3 mm², or 97.4±1.2% of the denuded area), whereas in the no-statin animals, reendothelialization remained limited to 14.1±0.6 mm², or 80.5±3.4% (P<0.05 versus statin group). Reendothelialization achieved with the higher dose of statin (1 mg/kg) was nearly complete at 2 weeks (data not shown). Representative macroscopic photographs of Evans blue dye–stained segments from statin-treated and control animals are shown in Figure 1B. In vivo BS1-lectin perfusion staining of longitudinal tissue sections confirmed these macroscopic findings (Figure 1C).

**Effect of Statin on Neointimal Proliferation**

Accordingly, the impact of statin therapy on neointimal thickening was studied in 52 normocholesterolemic (mean

(Caltag) were used. Phycoerythrin-conjugated antibodies were anti-CD31 (Pharmingen), anti-endothelial P1H12 (Chemicon), or AC133 (Miltenyi Biotech). Isotype-identical directly conjugated antibodies served as a negative control. HUVECs (passage 3) served as a positive control. Immunofluorescence-labeled cells were fixed with 2% paraformaldehyde and analyzed by quantitative flow cytometry using a FACStar flow cytometer (Becton Dickinson) and Cell Quest Software counting 10 000 events per sample.

**Statistical Analysis**

All data are presented as mean±SEM. Continuous variables were compared by Student’s t test or the Mann-Whitney U Test. Multiple comparisons were performed by Kruskal-Wallis test or ANOVA with Bonferroni’s correction using SPSS 9.0. A value of P<0.05 was considered significant.

**Results**

**Effect of Statin on Reendothelialization**

Planimetric analysis of rat carotid artery specimens documented that the total areas of initial balloon injury in the statin

**Figure 1.** Statins accelerate reendothelialization. A, Quantification of reendothelialized area expressed as mean±SEM (n=6 per study group). *P<0.01 simvastatin-injected animals (0.2 mg/kg) vs saline-injected; †P<0.05 simvastatin-injected animals (0.2 mg/kg) vs saline-injected. B, Macroscopic photomicrographs of Evans blue dye staining of whole-mounted carotid arteries 2 and 4 weeks after treatment initiation. White areas represent functionally intact endothelium (C); longitudinal sections after in vivo BS1-lectin perfusion for fluorescence microscopic identification of endothelium with statin (0.2 mg/kg) vs no-statin treatment at 2 weeks after balloon denudation.

**Figure 2.** Dose-dependent reduction in neointimal thickening. A, Male Sprague-Dawley rats were injected intraperitoneally with simvastatin 0.2 mg · kg⁻¹ · d⁻¹ (light blue line), 1 mg · kg⁻¹ · d⁻¹ (red line), or saline (dark blue line) after balloon denudation. ∗All values of P<0.01 statin vs saline, †P<0.05, statin 0.2 vs 1 mg/kg. B, Elastic tissue–stained histological cross sections indicating significant increase in neointimal thickening in non–statin-treated vs statin-treated animals (1 mg/kg) at 2 and 4 weeks. White arrows indicate internal elastic lamina.
Cholesterol Serum Levels

<table>
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<th>Control Rats (n=8)</th>
<th>Statin-Treated Rats (1 mg/kg) (n=8)</th>
<th>P</th>
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<tr>
<td>Baseline cholesterol</td>
<td>74.6±6</td>
<td>75.4±6</td>
<td>0.8</td>
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<td>Cholesterol (2 weeks)</td>
<td>75.4±6</td>
<td>74.6±4</td>
<td>0.8</td>
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<tr>
<td>Cholesterol (4 weeks)</td>
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<td>74±3</td>
<td>0.9</td>
</tr>
<tr>
<td>Maximum cholesterol change</td>
<td>−0.02</td>
<td>−0.02</td>
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Values are mg/dL, mean±SEM.

cholesterol 75.2±2.5 mg/dL) male Sprague-Dawley rats at 2, 3, and 4 weeks after carotid injury (Figure 2). In saline-injected control rats (n=18), neointimal thickness (I/M) ratio increased markedly at 2 weeks (I/M ratio 1.76±0.11), 3 weeks (2.32±0.13), and 4 weeks (2.46±0.20), respectively. Statin therapy, however, resulted in a dose-dependent (0.2 or 1 mg/kg IP), statistically significant reduction in neointimal thickening at all time points compared with controls (Figure 2). I/M ratios of animals treated with simvastatin 0.2 mg/kg (n=18) were 1.32±0.10, 1.85±0.16, and 1.94±0.10, all P<0.05 versus control animals. Animals treated with the higher dose of simvastatin (1 mg/kg, n=16) demonstrated a further reduction in I/M ratio at 2 weeks (I/M ratio 1.04±0.10), 3 weeks (1.35±0.13), and 4 weeks (1.45±0.10), all P<0.01 versus controls. Cholesterol levels were similar for treated and untreated animals (see the Table).

Effect of Statin on EPC Incorporation Into Balloon-Injured Carotid Artery

To assess the potential contribution of bone marrow–derived EPCs to accelerated reendothelialization, bone marrow from Tie2/lacZ mice was transplanted to background mice or nude rats, and carotid specimens were harvested 2 weeks after balloon injury. Quantification of whole-mounted X-gal–stained carotid arteries of mice revealed a 2.9-fold increase in the number of β-gal–positive cells/mm² appearing on the luminal surface in statin-treated animals (1 mg · kg⁻¹ · d⁻¹, n= 5) compared with controls (n=7) (43±2 versus 15±3 cells/mm², P<0.001) (Figure 3A).

Double-fluorescence immunohistochemistry to further identify bone marrow–derived Tie2/lacZ-positive endothelial cells disclosed a significant 5-fold increase in the number of double-positive cells on the reendothelialized luminal surface in cross sections of statin-treated (1 mg · kg⁻¹ · d⁻¹, n=6) versus saline-injected (n=6) nude rats (20±3 versus 4±1 cells/mm surface length, P<0.005). In cross sections of carotid arteries from the no-statin group, few cells stained positive for both β-gal and the endothelium-specific marker isolectin B4. In contrast, numerous double-positive cells were observed in carotid arteries from statin-treated animals (Figure 3, B and C). These data thus suggest that expedited reendothelialization achieved with statins involves augmented EPC incorporation into the carotid artery neointima.

Statins Increase Rat EPC Mobilization

To demonstrate that statin therapy increases the number of circulating EPCs, rat EPC culture assays were performed. Indeed, statins enhanced the number of circulating EPCs, as shown by costaining of cultured EPCs with DiI/acLDL and BS1-lectin. Before balloon denudation, isolation of blood mononuclear cells from both statin-treated and control rats revealed similar numbers of circulating EPCs (31±4 versus 30±4/mm², P=NS). At 2 and 4 weeks after balloon denuda-
tion, saline-injected rats showed no significant change in circulating EPCs (34±2/1,100/mm², P/N.S., n=6). Initiation of statin therapy (1 mg/kg), however, led to a 2.4-fold increase of EPCs at 2 weeks (78±1,100/mm², P/0.001, n=8); this effect was sustained through 4 weeks of statin treatment (2.5-fold increase, 80±7 EPCs/mm², P<0.001, n=8) (Figure 4).

**Effect of Statin on EPC Adhesiveness**

To study the possibility that statins alter adhesiveness of cultured human EPCs, 2 different adhesion assays were performed. Cultured human EPCs were incubated with simvastatin 1 μmol/L from day 4 to day 7. After replating on fibronectin-coated dishes, EPCs preexposed to simvastatin exhibited a significant increase in the number of adhesive cells at 30 minutes, from 46±5 to 65±7 (146±10% of controls, n=5, P<0.01).

In a second adhesion assay, the number of statin-treated versus control EPCs incorporating on a HUVEC monolayer also was shown to increase significantly (23±2 versus 13±2, 177±10%, P<0.01). In tumor necrosis factor-α-activated HUVECs, incorporation of statin-treated EPCs also exceeded that of untreated EPCs (36±3 versus 21±2, 172±10% of controls, P<0.01). These findings suggest that statins modulate the adhesiveness of EPCs to support homing to sites of vascular injury.

**Statin Modulation of Integrin Expression**

To evaluate statin modulation of integrin expression that might contribute to EPC neoendothelial incorporation, we performed semiquantitative RT-PCR of simvastatin-treated (3 days, 1 μmol/L) or untreated cultured human EPCs. Day 7 EPC cultures were used; cDNA from cultured HUVECs (passage 3) served as a positive control. Indeed, statins induced upregulation of integrin subunits α5 and β1, which compose the fibronectin receptor, and the αv and β3 subunits of human EPCs. The integrin subunit β3, part of the vitronectin receptor, was not expressed at the RNA level in day 7 EPC cultures. Statins also downregulated expression of VCAM-1, whereas expression levels of platelet and endothel...
cell adhesion molecule (CD31) were already comparable to those of HUVECs (Figure 5).

These data were further supported by FACS analysis (Figure 6), which disclosed that the majority of the EPCs expressed endothelial epitopes such as endothelium-specific P1H12 (15%) or CD31 (20%). Further characterization of surface antigens of cultured EPCs showed that a small percentage of cells were also positive for CD68 (15%) but were negative for AC133. By FACS analysis, simvastatin enhanced cell surface expression of integrin subunits as well as integrin receptor blockade abrogated the increased incorporation of EPCs into the neointima of balloon-injured carotid arteries as well as accelerated reendothelialization in response to statin therapy (1.5 versus 20.4-gal–positive cells/mm, P<0.001). Blocking experiments further confirmed that the enhanced contribution of EPCs to reendothelialization with statins inversely correlated with subsequent development of neointimal proliferation (r = −0.74, P<0.001) (Figure 7).

**Discussion**

The demonstration that statins accelerate reendothelialization constitutes a novel action for this class of agents. This results, at least in part, from the contribution of bone marrow–derived cells of endothelial lineage, which are mobilized in response to statin therapy, establishing physiological

**Figure 6.** Statin modulation of integrin expression (FACS analysis). A, Representative histograms of surface receptor expression. Gray line, mouse IgG control–labeled cells; blue line, untreated cells (no statin); and red line, cells exposed to simvastatin 1 μmol/L. Histograms display fluorescence intensity (x axis) vs relative cell number (y axis). Similar results were obtained in 5 additional experiments. B, Four-quadrant analysis of double-positive cells for endothelium-specific P1H12 (x axis) and integrin receptor expression (y axis) for untreated (blue) and statin-treated (red) EPCs. Double-positive cells appear in right upper quadrant. Quantification expresses relative cell number as percentage (mean ± SEM).
Evidence that EPC mobilization constitutes a functionally relevant consequence of statin therapy.

It is likely that statin-induced mobilization of EPCs also contributes to the previously described impact of statins on promoting tissue neovascularization.11 In this regard, statins share certain activities with vascular endothelial growth factor (VEGF),13 including the potential to promote reendothelialization13–16 and neovascularization11,12,28 and EPC mobilization in animals23,25 and human subjects undergoing VEGF gene transfer29 or in statin-treated patients with stable coronary artery disease.22 Indeed, recent work from our laboratory20 and others21 has demonstrated that these actions of statins, like those of VEGF,30–32 are mediated via phosphorylation of the serine/threonine protein kinase, Akt.

Homing to and incorporation into sites of reendothelialization probably is determined not only by the number of circulating EPCs but also by EPC maturation and/or differentiation. In this regard, the effects of statin therapy were not limited to augmented numbers of circulating EPCs. Enhanced adhesion of cultured human EPCs was demonstrated here in 2 different assays. Moreover, integrin receptor subunits α, β, α, and β were found to be upregulated, the functional relevance of which was confirmed by FACS analysis for both subunits of the classic fibronectin receptor α, β, and integrin receptor α, β. Modulation of integrin receptor expression may thus determine adhesiveness and thus promote homing of EPCs to foci of ischemia or vascular injury. Fibronectin, an extracellular matrix protein that may influence cellular migration and differentiation, accumulates rapidly at the site of balloon injury, an alteration of the vascular wall homing site that might be expected to facilitate EPC incorporation.33 Likewise, previous investigations have established the critical role of α, β in mediating the response of endothelial lineage cells to VEGF.26,34

These findings thus establish additional mechanisms by which statins may specifically preempt disordered vascular wall pathology and augment angiogenesis.

Figure 7. Inverse correlation between reendothelialization and intimal hyperplasia. A and B, EPC incorporation and I/M ratio (mean±SD) of carotid arteries from bone marrow–transplanted nude rats 2 weeks after denudation, detected by double-fluorescence labeling in rats injected with saline, treated with simvastatin, and coinjected with simvastatin and RGDfV peptide. *P<0.01 statin vs saline, IP=0.05 statin vs saline+RGDfV.

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References


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