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 ± 1.8 versus 5.4 ± 1.1 mm², 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 β-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 (β-gal, isolectin B4) on the luminal surface in carotid arteries of statin-treated nude rats (20 ± 3 versus 4 ± 1 cells/mm² 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 α₅, β₁, α₅, and β₃ 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.1 Moreover, primary and secondary prevention trials suggested, and laboratory investigations established, that statins possess favorable effects independent of cholesterol reduction.2,3 In particular, statins have been shown to decrease neointimal thickening in animal models of carotid injury4,5 and reduce clinical events and angiographic restenosis after coronary stent implantation.6

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These findings have been attributed, at least in part, to inhibition of vascular smooth muscle cell proliferation.7 Several groups, however, have demonstrated profound, positive effects of statins on endothelial cell function.8–10 Most recently, in vivo studies have established that statins may promote angiogenesis in ischemic limbs,11 analogously to endothelial cell mitogens.12 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.13–16

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 cells17,18 are capable of homing to sites of endothelial disruption and incorporating into nascent endothelium.19 More recently, 2 groups have documented in animals20,21 and human subjects22 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 determined at death.

Histological Analysis

To measure the reendothelialized area, animals were perfused either in vivo with Evans blue dye14 (Sigma) to identify the remaining denuded area or in vivo with BS1-lectin or isolectin 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 elastin trichrome stain.

Mouse and Rat Bone Marrow Transplantation Model

Mouse and rat bone marrow transplantation models were performed as previously described.23-25 In brief, lethally irradiated FVB/N mice (Jackson Laboratories, Bar Harbor, Me) or nude rats (Hsd:RH-nu rats, Harlan Sprague Dawley, Indianapolis, Ind) received bone marrow cells from transgenic Tie2/lacZ mice, which constitutively expressed β-galactosidase. Mouse and rat bone marrow transplantation with a wax balloon technique in bone marrow transplantation models was performed as previously described.22-25,27

Cell Adhesion Assay

After 3 days of incubation with simvastatin 1 μmol/L, human EPCs (day 7) were washed 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.

Incorporation Assay of EPCs Into 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 Dil for cell tracking (Biomedical Technologies). Identical numbers of Dil-labeled EPCs were incubated for 24 hours on a human umbilical vein endothelial cell (HUVEC) monolayer plated on fibronectin-coated 6-well culture dishes with or without pretreatment with tumor necrosis factor-α (1 ng/mL) for 12 hours. Nonadherent 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.

Semiquantitative Reverse Transcription–Polymerase Chain Reaction

Expression of the surface receptor integrin subunits αvβ3, αvβ5, 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 use of the Ambion RNAqueous kit. cDNA synthesis was performed with 1 μg of total RNA treated with DNase I (0.5 U/μg RNA) with the SuperScript II kit (Life Technologies) according to the manufacturer’s instructions. PCR conditions were 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds and 64°C for 3 minutes, and ending with 5 minutes of 64°C with Advantage-GC cDNA polymerase (Clontech). For semiquantification, QuantumRNA 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 imaging Eagle-Eye (Stratagene).

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 α6 (CD49E), β1 (CD29), anti-α,β1 (all Chemicon), anti-α,β1 (CD51/61, Pharmingen), or anti–VCAM-1 (CD 106, Chemicon) and anti-CD68

Fluorescence Immunohistochemistry of Carotid Arteries

The carotid arteries of transplanted nude rats (n = 12) were harvested at predetermined times after balloon injury, and double immunohistochemistry was performed with an antibody against β-galactosidase and isoleucin B4. Rabbit polyclonal anti-mouse β-galactosidase antibody (Cortex) was used at 1:200 dilution and 4°C overnight, followed by goat anti-rabbit IgG conjugated with Cy3 (Jackson Immunoresearch) as a secondary antibody at 1:400 dilution overnight. Endothelium-specific isoleucin B4 conjugated with fluorescein isothiocyanate (FITC; Vector) was used at 1:100 dilution and 4°C overnight. Rabbit IgG antibody served as a negative control. Double-positive cells that incorporated into the endothelial layer were counted in at least 10 different cross sections from different animals and expressed as average number per luminal surface length (in millimeters).

Rat EPC Culture

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/Dil (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.

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.24,25,27

Human EPCs were kindly provided by the laboratory of Dr. D. Moser (University of Bern, Switzerland) and were maintained in complete medium (10% FCS) in an incubator containing 5% CO2 at 37°C. EPCs were used between passages 3 and 8.

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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 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.

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.

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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.
To assess the potential contribution of bone marrow–

Balloon-Injured Carotid Artery

Effect of Statin on EPC Incorporation Into

Ballooned-Injured Carotid Artery

To evaluate the potential contribution of bone marrow–

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 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/mm² 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 aug-
mented EPC incorporation into the carotid artery
neoendothelium.

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 Dil/α-LDL 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±3 versus 31±3/mm², P=NS, n=6). Initiation of statin therapy (1 mg/kg), however, led to a 2.4-fold increase of EPCs at 2 weeks (78±6/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 endothelial cell adhesion molecule-1 (CD31) remained constant.

![Image](http://circ.ahajournals.org/)

**Figure 4.** Statins enhance number of circulating EPCs. Rat EPC culture assay indicating fluorescent Dil/acLDL BS1-lectin staining (>100). Quantification (mean±SEM) of Dil/BS1-lectin double-positive cells from rats injected with saline (blue line, n=6) or simvastatin (1 mg/kg) (red line, n=8) before and 2 or 4 weeks after balloon denudation. *All values of P<0.01, statin vs saline.*

![Image](http://circ.ahajournals.org/)

**Figure 5.** Statin modulation of integrin expression (mRNA). A, RT-PCR to identify expression of surface receptors from day 7 cultured human EPCs: Lane 1 (-S), control group EPCs. Lane 2 (+S), simvastatin-treated (1 μmol/L) EPCs. Lane 3 (H), HUVEC-positive control. Lane c, negative control. Semiquantification was performed using 18S standards. B, Data were quantified by UV imaging system and are expressed as percent increase of integrin mRNA/18S RNA. Similar results were obtained in 3 additional experiments.
lial 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 (75±4%) or CD31 (85±3%). Further characterization of surface antigens of cultured EPCs showed that a small percentage of cells were also positive for CD68 (15±3%) but were negative for AC133. By FACS analysis, simvastatin enhanced cell surface expression of α5β1 and α5β3 integrin subunits as well as αvβ3 integrin (Figure 6A). Percentage increases of double-positive EPCs for the endothelium-specific marker P1H12 and integrin subunits α5, β1, and αvβ3 are shown in Figure 6B.

To confirm the in vivo relevance of statin-mediated integrin upregulation, nude rats that had received bone marrow transplants were coinjected for 2 weeks after balloon injury with simvastatin and a cyclic RGD peptide directed against α5β1 and αvβ3 integrin receptors. Indeed, integrin receptor blockade abrogated the increased incorporation of EPCs into the neoendothelium of balloon-injured carotid arteries as well as accelerated reendothelialization in response to statin therapy (1.5±0.2 versus 20±3 β-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

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**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. In this regard, statins share certain activities with vascular endothelial growth factor (VEGF), including the potential to promote reendothelialization and neovascularization and EPC mobilization in animals and human subjects undergoing VEGF gene transfer or in statin-treated patients with stable coronary artery disease. Indeed, recent work from our laboratory and others has demonstrated that these actions of statins, like those of VEGF, 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 α5, β3, and αv were found to be upregulated, the functional relevance of which was confirmed by FACS analysis for both subunits of the classic fibronectin receptor αvβ3 and integrin receptor αvβ5. 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. Likewise, previous investigations have established the critical role of α5β3 in mediating the response of endothelial lineage cells to VEGF.

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