Statins Have Biphasic Effects on Angiogenesis

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Background—Statins inhibit HMG-CoA reductase to reduce the synthesis of cholesterol and isoprenoids that modulate diverse cell functions. We investigated the effect of the statins cerivastatin and atorvastatin on angiogenesis in vitro and in vivo.

Methods and Results—Endothelial cell proliferation, migration, and differentiation were enhanced at low concentrations (0.005 to 0.01 μmol/L) but significantly inhibited at high statin concentrations (0.05 to 1 μmol/L). Antiangiogenic effects at high concentrations were associated with decreased endothelial release of vascular endothelial growth factor and increased endothelial apoptosis and were reversed by geranylgeranyl pyrophosphate. In murine models, inflammation-induced angiogenesis was enhanced with low-dose statin therapy (0.5 mg · kg⁻¹ · d⁻¹) but significantly inhibited with high concentrations of cerivastatin or atorvastatin (2.5 mg · kg⁻¹ · d⁻¹). Despite the fact that high-dose statin treatment was effective at reducing lipid levels in hyperlipidemic apolipoprotein E–deficient mice, it impaired rather than enhanced angiogenesis. Finally, high-dose cerivastatin decreased tumor growth and tumor vascularization in a murine Lewis lung cancer model.

Conclusions—HMG-CoA reductase inhibition has a biphasic dose-dependent effect on angiogenesis that is lipid independent and associated with alterations in endothelial apoptosis and vascular endothelial growth factor signaling. Statins have proangiogenic effects at low therapeutic concentrations but angiostatic effects at high concentrations that are reversed by geranylgeranyl pyrophosphate. At clinically relevant doses, statins may modulate angiogenesis in humans via effects on geranylated proteins. (Circulation. 2002;105:739-745.)

Key Words: endothelium ■ inflammation ■ apoptosis ■ hypoxia ■ lipids

Angiogenesis, the sprouting of new capillaries from pre-existing blood vessels, is a multistep process that involves proliferation, migration, and differentiation of endothelial cells (ECs), remodeling of the extracellular matrix, and functional maturation of the newly assembled vessels.1,2 HMG-CoA reductase inhibitors, or statins, inhibit the biosynthesis of L-mevalonate. L-Mevalonate is a precursor for cholesterol, as well as isoprenoid intermediates such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGP).4 The isoprenoids are important lipid moieties added during posttranslational modification of a variety of proteins, including G-proteins and G-protein subunits, Ras, and Ras-like proteins, such as Rho, Rab, Rae, Ral, or Ra.4 Statin therapy effectively reduces cardiovascular events in patients at risk.3,5,6 The benefit appears to exceed the cholesterol-lowering effect of statins, possibly by protective effects on endothelial nitric oxide bioactivity and atherosclerotic plaque stabilization.5–7 Furthermore, it is possible that statins may modulate angiogenesis in a therapeutic manner. We and others have demonstrated that endothelial function and angiogenesis are impaired by hypercholesterolemia.8,9 By reducing cholesterol and enhancing endothelial functions, statins might improve angiogenesis. Accordingly, we hypothesized that HMG-CoA reductase inhibition would enhance endothelial processes involved in angiogenesis and might reverse impaired angiogenesis in hypercholesterolemia. Therefore, our studies were designed to determine the effect of HMG-CoA reductase inhibition on angiogenesis and its cellular determinants, as well as to determine the lipid dependency of these effects.

Methods

Reagents

Reagents were from Sigma unless otherwise specified. Cerivastatin (CEV) was supplied by Bayer AG, and atorvastatin (ATV) was a gift from U. Laufs (Homburg, Germany). Both drugs are potent inhibitors of HMG-CoA reductase.10,11

Cells

Primary human adult dermal microvascular ECs (HMVECs), obtained from BioWhittaker were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 0.1 mg/mL endothelial growth supplement (crude extract from bovine brain), 5 μg of human epidermal growth factor (hEGF), 0.5 mg of hydrocortisone, 50 U/mL penicillin, and 50 μg/mL streptomycin (Gibco BRL, Life Technologies). HMEC-1, an immortalized human dermal microvascular EC line, was provided by the Centers for Disease Control and Prevention, Atlanta, Ga.12 HMEC-1 cells were cultured in DMEM supplemented with 10% FBS, 10 ng/mL hEGF, 50 U/mL penicillin, and 50

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Assessment of Cell Number (XTT Assay)

HMVECs and HMEC-1 cells were seeded in 96-well plates (Falcon, 96 wells, flat bottom) at 80% confluence ($1 \times 10^5$ cells/well) and incubated in 2% FBS medium for 24 hours. CEV was added at concentrations ranging from 0.005 to 0.5 μmol/L. After 24 hours, XTT (Boehringer Mannheim; 125 mg/mL) was added to each well for 4-hour incubation, respectively. The antigen-antibody complexes were visualized with streptavidin/horseradish peroxidase diluted 1:1000 in 1% milk and 0.05% Tris-buffered saline-Tween 20. The antigen-antibody complex was visualized with streptavidin/horseradish peroxidase (Dako) diluted 1:500 and enhanced chemiluminescence detection.

Migration Assay (Scratch Wound Assay)

For detection of cell migration, cells were grown in pretracted 6-cm wells, and a portion of the cell monolayer was scraped away with a sterile disposable rubber policeman. The remaining cells were washed with medium and incubated with CEV or ATV (0.005 to 0.5 μmol/L) over 12, 36, and 60 hours. EC migration into the denuded area was quantified with a computer-assisted microscope (Olympus BX50F).

Apoptosis Assays (Annexin V and Sytox Staining)

HMVECs and HMEC-1 cells were grown in chamber slides (4% FBS) and exposed to hypoxia (2% O₂) for 24 hours with and without CEV. Nuclear fragmentation was quantified with Sytox (Molecular Probes) and a fluorescence microscope (Olympus BX50F) equipped with a 3-CCD color video camera (Sony DXC-970 MD) and the image software Optimas 6.1. Fluorescence-labeled annexin V (Molecular Probes) staining of HMEC-1 was used as a functional index of early apoptosis. Quantitative analysis of stained cells was performed in 5 random fields at 10-fold magnification (n=5).

Bax/Bcl-2 Protein Expression (Western Blot)

Cells were lysed by addition of 0.5 mL of Kephos buffer pH 7.0 including 1% NP-40, 1 μmol/L leupeptin, 5 μmol/L aprotinin, and 1 mmol/L 2-mercaptoethanol. Protein extracts (50 μg) were separated with 7.5% SDS-PAGE, transferred to a nitrocellulose membrane by electrotransfer, and blocked with 5% nonfat milk for 1 hour at room temperature. Bax and Bcl-2 were detected with rabbit anti-human antibody (Santa Cruz Biotechnology) diluted 1:1000 (2-hour incubation, respectively). The antibody-antibody complexes were visualized with anti-rabbit IgG horseradish peroxidase diluted 1:1000 and an enhanced chemiluminescence detection system (Amersham).

Endothelial Vascular Endothelial Growth Factor Release (ELISA)

Vascular endothelial growth factor (VEGF) levels in conditioned medium were detected with a human VEGF ELISA kit (Quantikine, R&D Systems). The VEGF standards (1 to 1000 pg/mL) and samples were placed by pipette into wells coated with antibody specific for human VEGF. After a wash, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. After a second wash, a substrate solution was added. The absorbance of standards and samples was measured spectrophotometrically at 450 nm with a microplate reader. VEGF concentrations were calculated (in pg/mL) with the standard curve and adjusted for protein concentrations.

VEGF Receptor-2 Expression

Blots were incubated with a monoclonal bixin-conjugated mouse anti-human VEGF receptor-2 (VEGFR-2) antibody (Sigma) diluted 1:1000 in 1% milk and 0.05% Tris-buffered saline-Tween 20. The antigen-antibody complex was visualized with streptavidin/horseradish peroxidase (Dako) diluted 1:500 and enhanced chemiluminescence detection.

In Vitro Angiogenesis

The formation of vascular-like structures by HMEC-1 cells was assessed on growth factor–reduced Matrigel (Becton Dickinson). HMEC-1 cells cultured for 24 hours in DMEM with 2% FBS were then plated at $2 \times 10^4$ cells/well in 4-well-chamber glass slides (Laboratory-Tek II; Nalge Nunc) precoated with 250 μL of Matrigel (10.7 mg/mL) in the absence or presence of CEV or ATV (0.005 and 0.5 μmol/L). After 48 hours of incubation in a 5% CO₂-humidified atmosphere at 37°C, tube formation was quantified by microscopy and a CCD camera. The length of completed tubelike structures in 5 random fields (10-fold magnification) was quantified in a blinded fashion.

Animals

Female wild-type and apolipoprotein (apo) E–deficient hypercholesterolemic C57BL/6J mice (apoE<sup>−/−</sup>; Jackson Laboratories, Bar Harbor, Me) aged 24 weeks were used to examine the angiogenic effects of CEV in normocholesterolemic and hypercholesterolemic states. Protocols were approved by the Administrative Panel on Laboratory Animal Care of Stanford University and were performed in accordance with their recommendations. Mice were treated with CEV (0.5 and 2.5 mg·kg<sup>−1</sup>·d<sup>−1</sup>) in drinking water or with daily subcutaneous injections of ATV (0.5 and 2.5 mg/kg). The dose range was based on biotransformation studies with CEV in mice<sup>13,16</sup> and on our studies of the lipid-lowering effects of statins in apoE<sup>−/−</sup> mice (~35% and 15% reduction in cholesterol levels after 7 days’ treatment with 2.5 or 0.5 mg·kg<sup>−1</sup>·d<sup>−1</sup> CEV). Serum cholesterol and triglyceride levels were determined by the Department of Comparative Medicine, Stanford University.

Figure 1. Biphasic effect of statin on EC proliferation. ECs were incubated with increasing concentrations of CEV (C) ranging from 0.001 to 1 μmol/L. CEV has a biphasic dose-dependent effect on proliferation of EC line HMEC-1. Mevalonate (Mev; 100 μmol/L) and GGP (20 μmol/L) but not FPP (20 μmol/L) or squalene (Squa; 100 μmol/L) reversed CEV-mediated inhibition of EC proliferation. Qualitatively identical results were obtained with primary HMVECs (data not shown).
In Vivo Angiogenic Assays

Tumor Angiogenesis (Lewis Lung Cancer Model)

Lewis lung cancer cells (LLC1; ATCC) were injected into both flanks of C57BL/6 mice. Mice were treated with CEV (0.5 or 2.5 mg·kg⁻¹·d⁻¹) in drinking water. Tumor growth was assessed by palpation 3 times per week. After 14 or 24 days, just before the animals were killed, 2 mL of fluorescent microspheres (0.2 μm; Molecular Probes) was injected into the left ventricle over 4 minutes. Tumor tissue was explanted, measured, weighed, and embedded in Tissue Tek at −80°C until sectioning. The long and short axes of each tumor were measured with calipers. The volume was calculated by the formula \((0.52)(ab^2)\), with \(a\) as the larger diameter and \(b\) as the smaller diameter. Tumor vessels in frozen mid-tissue sections (6 sections per tumor) were studied under a fluorescence microscope. Tumor vascularization was quantified by analysis of the vessel density in 5 random fields from each tumor section in a blinded fashion with Scion Image software (beta 4.0.2).

Inflammation-Induced Angiogenesis (Disk Model)

We have previously described the disk angiogenesis system. Disks were implanted into the subcutaneous flank in mice after anesthesia with intraperitoneal injection of xylcocaine/ketamine. Three weeks after disk implantation, and immediately after systemic infusion of fluorescent microspheres (0.2 μm; FluoSpheres carboxylate-modified microspheres, blue fluorescent; Molecular Probes) was injected into the left ventricle over 4 minutes. Tumor tissue was explanted, measured, weighed, and embedded in Tissue Tek (Sakura Finetek) at −80°C until sectioning. The long and short axes of each tumor were measured with calipers. The volume was calculated by the formula \((0.52)(ab^2)\), with \(a\) as the larger diameter and \(b\) as the smaller diameter. Tumor vessels in frozen mid-tissue sections (6 sections per tumor) were studied under a fluorescence microscope. Tumor vascularization was quantified by analysis of the vessel density in 5 random fields from each tumor section in a blinded fashion with Scion Image software (beta 4.0.2).

Statistical Analysis

Statistical analysis of the results was performed with the Student \(t\) test for unpaired or paired data, respectively, or, if necessary, with ANOVA followed by the Student-Newman-Keuls post hoc test (StatView 5.0). Data are presented as mean±SEM.

Results

EC Proliferation

EC proliferation (HMEC-1) was dose dependently affected by CEV (Figure 1). Low concentrations (0.01 to 0.005 μmol/L) increased cell proliferation by 10% to 20%, whereas high concentrations (≥0.05 μmol/L) decreased proliferation up to 38%. Mevalonate (100 μmol/L) and GGP (20 μmol/L) but not FPP (20 μmol/L) or squalene (100 μmol/L) reversed the inhibitory effects of CEV. Qualitatively identical results were obtained with primary HMVECs (data not shown).

EC Migration

Low statin concentrations (0.005 μmol/L) increased migration distance (ATV up to 54±7% and CEV up to 75±9%), whereas intermediate and high concentrations (0.05 and 0.5 μmol/L) inhibited cell migration by up to 66±5% (CEV) and 51±4% (ATV) compared with control (Figure 2). Cotreatment of ECs with mevalonate (100 μmol/L) or GGP but not FPP completely reversed the effect of high statin concentrations.

EC Apoptosis

As shown in Figure 3A, low-dose CEV (0.005 μmol/L) tended to protect microvascular ECs from hypoxia-induced apoptosis (25±13% decrease in annexin V–positive cells;
VEGFR-2 protein expression under hypoxic conditions by 23% (apoE−/−) and 42% (apoE+/+) mice (26% vessel area versus 43%; P<0.01, respectively). The effect of high-dose CEV on tumor growth was associated with a 51% reduction in tumor vascularization (Figure 5B). Proliferation of LLC1 in vitro was not affected by high-dose CEV (data not shown).

Inflammation-Induced Angiogenesis
Disk vascular ingrowth after 21 days was enhanced in C57BL/6 mice treated with low-dose CEV and ATV (24% and 30% increase in vessel area versus control; P<0.05), whereas high-dose statins inhibited disk vascularization by 57% (CEV) and 70% (ATV), respectively (P<0.001). Disk vascularization was reduced in hypercholesterolemic (apoE−/−) mice (26% vessel area versus 43%; P<0.01), an effect that was partially reversed by low-dose statin treatment. Despite the fact that high-dose CEV was more effective than low-dose CEV at reducing lipid levels in hyperlipidemic apoE knockout mice (51% versus 31% reduction in cholesterol concentration; 33% versus 23% reduction in triglycerides; both P<0.05), it impaired rather than enhanced angiogenesis. Representative pictures of the disk vascular ingrowth in apoE−/− mice are shown in Figure 6.

Discussion
This is the first study to investigate the effect of HMG-CoA reductase inhibition on angiogenesis in vitro and in vivo over a broad (therapeutically relevant) concentration range. We were able to demonstrate biphasic dose-dependent, lipid-independent effects of HMG-CoA reductase inhibition on

P=0.058), whereas annexin V–positive cells were increased by 62±12% in cells treated with 0.5 μmol/L CEV. Accelerated apoptosis by high-dose CEV was completely blocked by mevalonate (100 μmol/L). Similarly, nuclear fragmentation (identified with Sytox) was increased in cells treated with 0.5 μmol/L CEV, whereas low-dose CEV decreased nuclear fragmentation (data not shown). Necrosis was not enhanced at the time that apoptosis was detected (data not shown).

Bax expression was increased by hypoxia and by high-dose statin. The antiapoptotic Bcl-2 protein was downregulated by high-dose CEV in normoxic and hypoxic conditions. Therefore, the Bax/Bcl-2 ratio significantly increased in ECs exposed to high-dose CEV, in normoxic and hypoxic conditions.

VEGFR-2 Expression
Low concentrations of CEV increased endothelial VEGF release during normoxic and hypoxic conditions by 13±6% (P<0.05) and 30±6% (P<0.01), respectively. In contrast, VEGF release from hypoxic ECs exposed to high-dose CEV (0.5 μmol/L) was significantly decreased compared with control (−23±9%; P<0.01). Mevalonate and GGP but not FPP reversed the inhibitory effects of high-dose CEV on VEGF release.

The effect of VEGF is mediated in part through endothelial VEGFR-2. Because Rho proteins may modulate endothelial VEGFR-2 expression, and Rho activity is modulated by products from the mevalonate pathway, we studied VEGFR-2 expression after CEV treatment. VEGFR-2 expression in hypoxic cells was significantly upregulated compared with normoxic cells. High-dose CEV (0.5 μmol/L) reduced VEGFR-2 protein expression under hypoxic conditions by 68±6% (P<0.001).

In Vitro Angiogenesis (Matrigel Assay)
The formation of vascular-like structures by HMEC-1 was strongly enhanced by low-dose (0.005 μmol/L) CEV (+360%) and ATV (+270%) but was impaired by high-dose (0.5 μmol/L) statin treatment (−61% and −52% in response to CEV and ATV, respectively; Figure 4). Mevalonate and GGP restored tube formation.

Tumor Angiogenesis
Tumor growth was comparable in the CEV and control groups 14 days after tumor cell injection (Figure 5A). After 24 days, tumor volume and weight were significantly reduced in C57BL/6 mice treated with high-dose CEV (2.5 mg · kg⁻¹ · d⁻¹; tumor volume −36%, tumor wet weight −59% versus control; P<0.01 and P<0.001, respectively). The effect of high-dose CEV on tumor growth was associated with a 51±17% reduction in tumor vascularization (Figure 5B). Proliferation of LLC1 in vitro was not affected by high-dose CEV (data not shown).

Figure 4. Effect of statins on EC differentiation. Low concentrations of statins (0.005 μmol/L CEV [C] or ATV [A]) enhanced tube formation. High-dose statin treatment (0.5 μmol/L) inhibited tube formation, an effect reversed by mevalonate (Mev) and GGP but not by FPP. Representative formation of capillary-like structures on growth factor–reduced Matrigel after 24-hour treatment with ATV is depicted in upper right panels.
angiogenesis. Moreover, we present new data that high-dose statin monotherapy reduces tumor growth, an effect associated with decreased tumor vascularization. Finally, this is the first study to demonstrate that HMG-CoA reductase inhibition dose dependently affects apoptotic activity and VEGF signaling in human ECs. The angiostatic effects of statins at high therapeutic concentrations are lipid independent and are reversible by supplementation with mevalonate and GGP. This observation indicates that angiogenesis may be impaired by inhibition of isoprenylation (geranylated proteins).

Serum levels of statins in humans range between 0.002 and 0.05 μmol/L for CEV (0.2 to 0.8 mg/d) \(^{10,19}\) and between 0.002 and 0.2 μmol/L for ATV (10 to 80 mg/d). \(^{20}\) In our in vitro studies, proangiogenic effects were observed at statin concentrations between 0.005 and 0.05 μmol/L (low- to mid-range concentrations in humans), whereas angiostatic effects were observed at ≥0.05 μmol/L CEV or ATV (high-dose concentrations in humans). Similarly, we chose to investigate the effect of a clinically relevant range of low- and high-dose statins in our murine models. The dosing was based on biotransformation studies in mice (which indicate accelerated disposition of statins) and the effect on lipid levels in the apoE-deficient mice. The in vitro and in vivo studies are concordant in demonstrating a biphasic effect of statins on angiogenesis that is dose dependent.

The existing literature regarding the effect of statins on angiogenesis has been puzzling. There are reports that statins can reduce EC proliferation and migration \(^ {21-23}\) and enhance the antitumor effect of tumor necrosis factor-α. \(^ {24}\) In contrast, Kureishi et al. \(^ {25}\) reported that simvastatin promotes angiogenesis in normocholesterolemic animals, probably via activation of endothelial protein kinase Akt/PKB. The discrepant data may result from the different EC types used or may be attributed to the different statin concentrations and incubation times.

Low-dose statin may enhance angiogenesis via activation of endothelial NO synthase. \(^ {25}\) NO promotes angiogenesis by enhancing EC proliferation, migration, and podokinesis. \(^ {26}\) By contrast, high-dose statins decreased angiogenesis. Our data indicate that at high doses, a statin-induced reduction in GGP inhibits angiogenesis. GGP is required for the membrane localization of small GTP-binding proteins such as Rho.
family members. Other antiangiogenic effects of statins may include inhibition of the expression or activity of monocyte chemoattractant protein-1, metalloproteinase and angiotensin-2, preproendothelin gene, and actin filament and focal adhesion formation.

EC survival factors are involved in angiogenesis. Dysfunction of Rho and Ras induces apoptosis. Our data indicate that high-dose statins increase EC apoptosis under hypoxic conditions. We speculate that high-dose statins induce EC apoptosis in part by reducing geranylgeranylation of Rho proteins known to modulate the activity of VEGFR-2. Similarly, Knapp et al. reported that simvastatin (2 μmol/L) increased cytokine-induced EC apoptosis. By contrast, Kureishi et al. demonstrated that simvastatin (1 μmol/L) decreased endothelial apoptosis under normoxic conditions. The differences between the studies may be explained by different EC types and different apoptotic stimuli used.

We have previously shown that hypercholesterolemia impairs angiogenesis, an effect that is mediated at least in part by an accumulation of asymmetric dimethylarginine, the endogenous inhibitor of NO synthesis. To define the contribution of reduced cholesterol synthesis to the angiogenic effects of statins, we investigated the effect of low- and high-dose statin treatment on angiogenesis in normal and genetically hypercholesterolemic mice. In the hypercholesterolemic group, angiogenesis was inhibited. Low-dose statin therapy enhanced angiogenesis in both groups. By contrast, high-dose cerivastatin inhibited angiogenesis in both groups, even though it was much more effective at reducing lipid levels in the apoE-deficient mice. Thus, the observed effects of HMG-CoA reductase inhibition on angiogenesis are primarily dependent on the absence of mevalonate but may not be entirely related to cholesterol reduction.

The aggressive growth of tumors is dependent on angiogenesis. We found that high-dose CEV decreased tumor vascularization and tumor growth. Blais et al. recently explored the association between statins and cancer incidence in humans. Patients treated with statins were found to be 28% less likely than users of bile acid–binding resins to be diagnosed as having any cancer (risk ratio 0.72; 95% CI 0.57 to 0.92). In the Scandinavian Simvastatin Survival Study trial, fewer cancer-related deaths were observed in patients receiving long-term simvastatin therapy.

Plaque growth is also dependent on angiogenesis. Proangiogenic agents such as VEGF and nicotine increase plaque neovascularization and progression. Accordingly, it is possible that the known benefit of statins on the progression of coronary atherosclerosis is due in part to inhibition of plaque neovascularization.

In summary, HMG-CoA reductase inhibition has a biphasic and dose-dependent effect on angiogenesis that is mainly independent of its effects on blood cholesterol and that is related to geranylated proteins. Our observation that angiogenesis is modulated by the HMG-CoA reductase pathway may establish a mechanistic basis for some of the lipid-independent effects of statins on cancer mortality and cardiovascular events.

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