3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitors Interfere With Angiogenesis by Inhibiting the Geranylgeranylation of RhoA

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Abstract—Angiogenesis is implicated in the pathogenesis of cancer, rheumatoid arthritis, and atherosclerosis and in the treatment of coronary artery and peripheral vascular disease. Here, cholesterol-lowering agents, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are shown to interfere with angiogenesis. In vivo, the HMG-CoA reductase inhibitor simvastatin dose-dependently inhibited capillary growth in both vascular endothelial growth factor–stimulated chick chorioallantoic membranes and basic fibroblast growth factor–stimulated mouse corneas. In vitro, the development of tubelike structures by human microvascular endothelial cells cultured on 3D collagen gels was inhibited at simvastatin concentrations similar to those found in the serum of patients on therapeutic doses of this agent. HMG-CoA reductase inhibitors interfered with angiogenesis via inhibition of the geranylgeranylation and membrane localization of RhoA. Simvastatin inhibited membrane localization of RhoA with a concentration dependence similar to that for the inhibition of tube formation, whereas geranylgeranyl pyrophosphate, the substrate for the geranylgeranylation of Rho, reversed the effect of simvastatin on tube formation and on the membrane localization of RhoA. Furthermore, tube formation was inhibited by GGTI, a specific inhibitor of the geranylgeranylation of Rho; by C3 exotoxin, which inactivates Rho; and by the adenoviral expression of a dominant-negative RhoA mutant. The expression of a dominant-activating RhoA mutant reversed the effect of simvastatin on tube formation. Finally, HMG-CoA reductase inhibitors inhibited signaling by vascular endothelial growth factor, Akt, and focal adhesion kinase, three RhoA-dependent pathways known to be involved in angiogenesis. This study demonstrates a new relationship between lipid metabolism and angiogenesis and an antiangiogenic effect of HMG-CoA reductase inhibitors with possible important therapeutic implications. (Circ Res. 2002;91:143-150.)

Key Words: 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors ■ angiogenesis ■ RhoA

Angiogenesis has been implicated in the pathogenesis of cancer, rheumatoid arthritis, diabetic retinopathy, and atherosclerosis.1 Furthermore, angiogenesis may play a role in the treatment of coronary artery and peripheral vascular disease.2 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, commonly referred to as statins, are in wide use for the treatment of hypercholesterolemia,3,4 which often coexists with vascular disease and other conditions whose pathogenesis may be dependent on angiogenesis. For this reason, intense interest has developed in studying the effect of HMG-CoA reductase inhibitors on angiogenesis.

HMG-CoA reductase inhibitors decrease LDL cholesterol by inhibiting the rate-limiting enzyme in cholesterol biosynthesis.3,4 Increasing evidence suggests that cholesterol lowering alone does not account for the therapeutic effects of statins.5,6 Thus, HMG-CoA reductase inhibitors have been shown to increase the production of NO and inhibit vascular smooth muscle cell proliferation, both of which might interfere with atherogenesis.7 It has been suggested that these effects involve the inhibition of the posttranslational lipidation of RhoA, a small GTP-binding protein. HMG-CoA reductase inhibitors could interfere with the biosynthesis of farnesyl pyrophosphate (FPP), which not only is a precursor of cholesterol, but also is required for the posttranslational lipidation of Ras. FPP condenses with isopentenyl pyrophosphate, whose synthesis is also blocked by HMG-CoA reductase inhibitors, to form geranylgeranyl pyrophosphate (GGPP), which is required for the posttranslational lipidation, membrane localization, and function of RhoA.3

Recent data have demonstrated that several signaling pathways that play a role in angiogenesis are dependent on RhoA. The initial step in vascular endothelial growth factor (VEGF) stimulation of angiogenesis is the VEGF-stimulated tyrosine phosphorylation of the VEGF receptor, flk-1/KDR.

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The expression of a dominant-activating (DA) mutant of RhoA has been shown to stimulate the tyrosine phosphorylation of flk-1/KDR, consistent with a role of RhoA in the activation of flk-1/KDR. The activation of focal adhesion kinase (FAK), which plays a role in cytoskeletal reorganization and cell migration, has also been shown to be dependent on RhoA. Finally, the stimulation of Akt kinase, which mediates increased endothelial cell survival, has been shown to be dependent on members of the Rho family of GTPases. In the present study, we present data demonstrating that HMG-CoA inhibitors interfere with new blood vessel formation in both in vitro and in vivo models of angiogenesis. We further demonstrate that tube formation by human vascular endothelial cells cultured on a 3D collagen gel is RhoA dependent and that HMG-CoA reductase inhibitors interfere with tube formation via the inhibition of the geranylgeranylation and membrane localization of RhoA and by interfering with RhoA-dependent cell-signaling pathways.

Materials and Methods
An expanded Materials and Methods section, including in vivo and in vitro angiogenesis assays, can be found in the online data supplement available at http://www.circresaha.org.

Localization of RhoA
For determination of membrane localization of RhoA, human dermal microvascular endothelial cells (HDMECs) were cultured with 2% FCS, and growth factors (40 ng/mL VEGF, 40 ng/mL basic fibroblast growth factor [FGF], and 50 ng/mL phorbol 12-myristate 13-acetate [PMA]) were added and treated with the indicated concentrations of simvastatin for 5 days. Cells were washed with PBS, resuspended in buffer (10 mmol/L Tris-HCl [pH 7.5], 1 mmol/L EDTA, 1 mmol/L EGTA, 0.1 mmol/L dithiothreitol, and protease inhibitor cocktail [1:50]), and sonicated on ice three times for 5 seconds, and the homogenate was separated into cytoplasmic (S100) and membrane (P100) fractions by ultracentrifugation at 100,000g for 40 minutes. The membrane fractions were solubilized in buffer, 50 mmol/L Tris-HCl (pH 7.5), 0.15 mol/L NaCl, 0.1 mmol/L dithiothreitol, 1% SDS, 1 mmol/L EDTA, 1 mmol/L EGTA, and protease inhibitor cocktail, followed by PAGE and immunoblot analysis with the use of an anti-RhoA antibody. Where indicated, FPP or GGPP was added to the culture 2 hours before harvesting the cells.

Adenoviral Infection of Endothelial Cells
Recombinant adenovirus encoding either a chimeric tetracycline transactivator or a dominant-negative (DN)-RhoA or a DA-RhoA under the control of 7 tetracycline operator sites has been described previously. HDMECs were cultured on collagen gels to near confluence in growth medium plus 10% FCS. Medium was removed, and cells were infected for 2 hours with virus in medium with 2% FCS at the indicated multiplicity of infection (MOI). Virus encoding a tetracycline transactivator alone was used at an MOI of 1:120, and adenovirus expressing DN-RhoA was used at an MOI of 1:20. For studies of the effect of DA-RhoA, simvastatin was added 24 hours after infection. After 4 or 5 days in culture, cells were stained as described, and tube formation was determined.

Results
Simvastatin Inhibits Growth Factor–Stimulated Angiogenesis In Vivo
To assess the effect of HMG-CoA reductase inhibitors on angiogenesis, we performed two well-established in vivo angiogenesis assays. In a mouse corneal pocket assay for angiogenesis, insertion of a pellet containing FGF-2 into the corneal pocket stimulated capillary growth into the cornea from the surrounding limbal vessels. This effect was accompanied by an intense hyperemia (Figure 1A, panel 1). Addition of the HMG-CoA reductase inhibitor simvastatin plus FGF-2 to the pellet markedly suppressed FGF-2–stimulated blood vessel growth, as demonstrated by the complete disappearance of both the new blood vessels and the hyperemic response (Figure 1A, panel 2). Of 29 corneas treated with FGF-2, 28 demonstrated an angiogenic response to FGF-2. Of the 29 corneas treated with FGF-2 plus 5 μmol/L simvastatin per pellet, the angiogenic response was abolished in 26 corneas. Microscopic examination demonstrated that FGF-2 resulted in the marked proliferation of tiny capillaries into the cornea (3, control; 4, FGF-2 alone; 5, FGF-2 plus 5 μmol/L simvastatin per pellet; and 6, FGF-2 plus 10 μmol/L simvastatin per pellet). B, VEGF-stimulated angiogenesis in a CAM assay: quantification of the angiogenic response in CAMs incubated with a vinyl mesh containing simvastatin alone (10 μmol/L), VEGF alone (250 ng), or VEGF plus the indicated concentrations of simvastatin. CAMs were studied as described in Materials and Methods. The data are from 4 independent experiments, each carried out in triplicate.
Apoptosis assay is shown. HDMECs were cultured on gelatin-coated dishes treated with various concentrations of simvastatin for 5 days, and cell death was quantified by measurements of cytoplasmic histone-associated DNA fragments as described in Materials and Methods. D, Quantification of the effect of FPP and GGPP on simvastatin inhibition of tube formation is shown. C indicates control; S, 0.5 μmol/L simvastatin; FPP, 10 μmol/L FPP; and GGPP, 10 μmol/L GGPP. Data are the mean of the total number of branch points in 8 low-power fields (>100) per well from 3 replicate wells. Data are presented as a percentage of control and are typical of 4 similar experiments. C, pellet (Figure 1A, panels 3, 4, and 6: 5 of 5 corneas). At 10 μmol/L simvastatin per pellet, only normal-appearing limbic vessels remained (panels 3 and 6).

In a chick chorioallantoic membrane (CAM) assay for angiogenesis, a matrix-polymer containing VEGF placed on the CAM stimulated capillary growth into the matrix. Quantitative analysis demonstrated that inclusion of VEGF stimulated angiogenesis 2.7-fold over control (Figure 1B, lanes 1 and 2). Simvastatin alone had no effect on the basal level of capillary vessel formation (Figure 1B, lanes 1 and 3). However, the incorporation of VEGF plus simvastatin into the matrix-polymer suppressed VEGF-stimulated capillary growth in a dose-dependent manner (lanes 4 through 8).

Simvastatin (1 μmol/L per pellet) decreased VEGF-stimulated angiogenesis by 30±5% (P<0.05; lanes 2 and 6, n=12), whereas 10 μmol/L simvastatin per pellet decreased the VEGF response to a level not significantly different from control (Figure 1B, lanes 1 and 8). Hence, simvastatin inhibited capillary blood vessel formation in response to two different growth factors in two in vivo angiogenesis assays.

Figure 2. Effect of simvastatin on tube formation by HDMECs cultured on 3D collagen gels. A, HDMECs were cultured with FGF (40 ng/mL) alone (panel 1); FGF, PMA (50 ng/mL), and VEGF (40 ng/mL) (panel 2); or FGF, PMA, and VEGF plus 0.1 μmol/L simvastatin (panel 3), 0.5 μmol/L simvastatin (panel 4), 0.5 μmol/L simvastatin plus 10 μmol/L FPP (panel 5), or 0.5 μmol/L simvastatin plus 10 μmol/L GGPP (panel 6) added at the time of plating. Incubation was continued for 4 or 5 days. Photomicrographs are typical of 4 independent experiments, each carried out in triplicate. Note the presence of elongated endothelial cells in the plane above the tubes. B, Dose-response curve for simvastatin inhibition of tube formation is shown. Cells were incubated as shown in panel A with growth factors and the indicated concentrations of simvastatin. Data are the mean of the total number of branch points in 8 low-power fields (>100) per well from 3 replicate wells. Data are presented as a percentage of control and are typical of 4 similar experiments.

Effect of HMG-CoA Reductase Inhibitors on Tube Formation by HDMECs Cultured on a 3D Collagen Matrix

To study the effect of HMG-CoA reductase inhibitors in vitro, we used a 3D collagen gel assay in which HDMECs were cultured on a thick collagen matrix. Long-term survival of HDMECs, which grow as a confluent monolayer of cobblestone-like cells on the surface of the gel, was dependent on the presence of FGF (Figure 2A, panel 1). However, in the presence of FGF, PMA and VEGF cells on the gel surface became elongated, invaded the collagen gel, and differentiated to form tubes that could be visualized by focusing below the surface of the gel (Figure 2A, panel 2). Simvastatin gave a dose-dependent inhibition of tube formation with a decrease of 30±7% (P<0.05, n=24) at 0.05 μmol/L, a decrease of 62±5% (P<0.01, n=24) at 0.1 μmol/L, and complete inhibition of tube formation at 0.5 μmol/L (Figure 2B) but did not interfere with the formation of elongated cells on the surface of the collagen (Figure 2A, panels 1 through 4). Mevastatin, an HMG-CoA reductase inhibitor, interfered with tube formation by HDMECs with a dose dependence similar to that for simvastatin (data not shown). To determine whether simvastatin inhibition of tube formation by HDMECs was due to an increase in apoptosis, HDMECs were incubated on gelatin-coated dishes in the presence of increasing concentrations of simvastatin under the same culture conditions and over the same time course that were used in the tube formation assay, and the effect of simvastatin on cell death was determined. A concentration of 0.1 μmol/L simvastatin, which inhibited tube formation by 62±5% (Figure 2B, lane 4), did not increase apoptosis above basal levels (Figure 2C). Furthermore, 0.5 μmol/L simvastatin, a concentration that inhibited tube formation completely, increased apoptosis only slightly above the basal level. Only at 5 μmol/L simvastatin did apoptosis increase significantly.

Simvastatin Inhibits Angiogenesis by Interfering With the Geranylgeranylation of RhoA

Because HMG-CoA reductase inhibitors interfere with the biosynthesis of FPP and GGPP, one mechanism by which they might inhibit angiogenesis is via the inhibition of protein lipidation. Incubation of cells with simvastatin plus FPP demonstrated no effect on simvastatin inhibition of tube formation (Figure 2A, panels 4 and 5; Figure 2D, lanes 2 and
Effects of Simvastatin on Membrane Localization of RhoA

To determine whether there was a correlation between simvastatin inhibition of tube formation and the inhibition of the membrane localization of Rho, HDMECs were incubated under the same culture conditions and over the same time course as used in the tube formation assays in Figure 2A, and the effect of increasing concentrations of simvastatin on the distribution of RhoA between the membrane and cytoplasm was determined. Simvastatin mediated a dose-dependent decrease in the membrane localization of RhoA and a parallel dose-dependent increase in cytoplasmic RhoA (Figure 4). This effect was significant at 0.1 μmol/L simvastatin, a concentration at which tube formation was inhibited by 62±5%, and even more marked at 0.5 μmol/L simvastatin, a concentration that completely inhibited tube formation. Furthermore, GGPP, which reversed the effect of simvastatin on tube formation, completely reversed the effect of simvastatin on the membrane localization of RhoA, whereas FPP, which had no effect on tube formation, had no effect on the localization of RhoA (Figure 4).

Effect of DN-RhoA Mutant on Angiogenesis In Vitro

To further establish the role of RhoA in angiogenesis, HDMECs were coinfected with an adenovirus expressing a DN-RhoA mutant under the control of a tetracycline transactivator and an adenovirus constitutively expressing the transactivator, and the effect on tube formation was determined. Infection of HDMECs with the adenovirus constitutively expressing the transactivator alone had no effect on tube formation (Figure 5A, panels 1 and 2; Figure 5C, lanes 1 and 2). Infection with the adenovirus expressing the DN-RhoA mutant alone decreased tube formation by 25±4% (P<0.01, n=15) compared with the control condition (Figure 5A, panels 2 and 3; Figure 5C, lanes 2 and 3). However, coinfection of cells with adenoviruses expressing the DN-RhoA and the transactivator decreased tube formation by 80±6% (P<0.01, n=15) compared with the control condition (Figure 5A, panels 2 and 4; Figure 5C, lanes 2 and 4). Under these conditions, the DN-RhoA was expressed at high levels (Figure 5B). These effects were specific for the inhibition of tube formation, as infection did not affect the morphology of the elongated endothelial cells on

Figure 3. Effect of FTI, GGTI, and C3 exotoxin on tube formation. A, HDMECs were cultured with FGF alone (panel 1); with growth factors plus 0.5 μmol/L simvastatin (panel 3); 10 μmol/L FTI (panel 4); 10 μmol/L GGTI (panel 5); or 0.5 μg/mL C3 exotoxin (panel 6). Photomicrographs are typical of 3 independent experiments, each carried out in triplicate. B, Quantification of the effect of FTI and GGTI on tube formation is shown. Data are the mean number of branch points in 8 low-power fields in each of 3 replicate wells similar to those in panel A (panels 2, 4, 5, and 6) and are plotted as percentage of control. Data are typical of 3 similar studies.

Figure 4. Effect of simvastatin on the membrane localization of RhoA. HDMECs were cultured in 2% medium in serum with growth factors and treated with the indicated concentrations of simvastatin for 5 days. FPP (F) or GGPP (G) was added at 10 μmol/L 2 hours before harvest, and RhoA was determined in S100 and P100 fractions by SDS-PAGE, followed by immunoblot analysis with use of an anti-RhoA antibody. These data are typical of 3 independent experiments.

Effect of DN-RhoA Mutant on Angiogenesis In Vitro

To further establish the role of RhoA in angiogenesis, HDMECs were coinfected with an adenovirus expressing a DN-RhoA mutant under the control of a tetracycline transactivator and an adenovirus constitutively expressing the transactivator, and the effect on tube formation was determined. Infection of HDMECs with the adenovirus constitutively expressing the transactivator alone had no effect on tube formation (Figure 5A, panels 1 and 2; Figure 5C, lanes 1 and 2). Infection with the adenovirus expressing the DN-RhoA mutant alone decreased tube formation by 25±4% (P<0.01, n=15) compared with the control condition (Figure 5A, panels 2 and 3; Figure 5C, lanes 2 and 3). However, coinfection of cells with adenoviruses expressing the DN-RhoA and the transactivator decreased tube formation by 80±6% (P<0.01, n=15) compared with the control condition (Figure 5A, panels 2 and 4; Figure 5C, lanes 2 and 4). Under these conditions, the DN-RhoA was expressed at high levels (Figure 5B). These effects were specific for the inhibition of tube formation, as infection did not affect the morphology of the elongated endothelial cells on
the gel surface (Figure 5A, panels 2 through 4). Because the expression of the DN-RhoA mutant is under the control of the tetracycline transactivator, the small decrease in tube formation in cells infected with an adenovirus expressing a tetracycline-controlled transactivator (2) or a DN-RhoA (3); and 4, cells coinfected with adenoviruses expressing a transactivator and a DN-RhoA. Cells were incubated, fixed, and stained as described in Materials and Methods. Photomicrographs are typical of 3 similar experiments. B, Immunoblot analysis of DN-RhoA expression. Cell extracts from infected cells were analyzed by using an anti-RhoA antibody. tTA denotes a tetracycline transactivator. These data are typical of 3 independent experiments. C, Quantification of the effects of a DN-RhoA mutant on tube formation by HDMECs. Data are presented as the mean number of branch points per 5 low-power fields from each of 3 replicate determinations plotted as the percentage of total tubes in control wells. Data are typical of 4 similar experiments.

Effect of DA-RhoA Mutant on Inhibition of Tube Formation by Simvastatin

If HMG-CoA reductase inhibitors interfere with angiogenesis via an effect on RhoA, then expression of a DA-RhoA mutant might reverse the effect of HMG-CoA reductase inhibitors on tube formation. Because the DA-RhoA requires geranylgeranyl to localize to the membrane, experiments were carried out at 0.05 μmol/L simvastatin, a concentration that inhibited tube formation by HDMECs to 47±10% of control (Figure 6A, panels 1 and 2; Figure 6B, lanes 1 and 2). Infection of HDMECs with an adenovirus expressing the tetracycline transactivator alone actually increased the inhibition of tube formation slightly. However, this change was not statistically significant (Figure 6A, panels 1 through 3; Figure 6B, lanes 1 through 3). Infection with an adenovirus expressing DA-RhoA alone resulted in a reversal of simvastatin inhibition of tube formation (Figure 6A, panels 2 and 4). This reversal of simvastatin inhibition of tube formation was dose dependent, from 47±10% of control to 86±10% (n=15, P<0.05) of control at an MOI of 10 (Figure 6B, lanes 4 through 6). This result was consistent with a significant dose-dependent increase in the basal expression of the DA-RhoA mutant, which increased with increasing MOI, as measured by use of an anti-myc antibody (Figure 6C).
conclusion that both basal and VEGF-stimulated phosphorylation of FAK below control levels, consistent with the conclusion that simvastatin inhibition of tube formation is due to the inhibition of RhoA function.

**Effects of HMG-CoA Reductase Inhibitors on RhoA-Dependent Signaling Pathways Involved in Angiogenesis**

To determine whether the antiangiogenic effect of HMG-CoA reductase inhibitors was associated with the inhibition of Rho-dependent signaling pathways involved in angiogenesis, we studied the effect of HMG-CoA reductase inhibitors on VEGF-stimulated phosphorylation of the VEGF receptor flk-1/KDR, FAK, and Akt. Simvastatin inhibited VEGF-stimulated tyrosine phosphorylation of flk-1/KDR in human umbilical vein endothelial cells (HUVECs) in a dose-dependent manner (Figure 7A, top) but had no effect on the expression of flk-1/KDR (Figure 7A, bottom). The HMG-CoA reductase inhibitor mevastatin also interfered with VEGF-stimulated tyrosine phosphorylation of flk-1/KDR in HUVECs (Figure 7B). Simvastatin inhibited VEGF-stimulated phosphorylation of flk-1/KDR in HUVECs with a dose dependence similar to that for the inhibition of tube formation (data not shown). C3 exotoxin mimicked the effect of simvastatin and mevastatin on VEGF-stimulated phosphorylation of flk-1/KDR in HUVECs, but had no effect on the expression of flk-1/KDR, consistent with its dependence on RhoA (Figure 7B). Under the conditions of this phosphorylation assay, simvastatin did not increase apoptosis in HUVECs above basal levels at concentrations as high as 0.5 μmol/L (data not shown).

Unlike flk-1/KDR, there was a significant level of tyrosine phosphorylation of FAK under control conditions. However, VEGF stimulated FAK phosphorylation significantly above control levels (Figure 7C, top, lanes 1 and 2). Both C3 exotoxin and simvastatin inhibited VEGF-stimulated phosphorylation of FAK below control levels, consistent with the conclusion that both basal and VEGF-stimulated phosphorylation of FAK are inhibited by HMG-CoA reductase inhibitors and are dependent on RhoA. C3 exotoxin and simvastatin decreased FAK expression slightly (Figure 7C, bottom).

Coinfection of HMECs with the adenoviruses expressing DA-RhoA plus the transactivator had no further effect on tube formation (data not shown). These results further support the conclusion that simvastatin inhibition of tube formation is due to the inhibition of RhoA function.

**Discussion**

These data demonstrate a new relationship between angiogenesis, lipid metabolism, and RhoA-dependent cell signaling. Thus, in three different model systems, HMG-CoA reductase inhibitors interfered with angiogenesis. In vivo, simvastatin inhibited VEGF-stimulated angiogenesis in the CAM assay and FGF-stimulated angiogenesis in the corneal pocket assay, and in vitro simvastatin and mevastatin inhibited tube formation by HMECs cultured on thick collagen gels. Simvastatin had no effect on the level of Akt expression.

Unlike flk-1/KDR, there was a significant level of tyrosine phosphorylation of FAK under control conditions. However, VEGF stimulated FAK phosphorylation significantly above control levels (Figure 7C, top, lanes 1 and 2). Both C3 exotoxin and simvastatin inhibited VEGF-stimulated phosphorylation of FAK below control levels, consistent with the conclusion that both basal and VEGF-stimulated phosphorylation of FAK are inhibited by HMG-CoA reductase inhibitors and are dependent on RhoA. C3 exotoxin and simvastatin decreased FAK expression slightly (Figure 7C, bottom).

Although we could not demonstrate a direct effect of an 18- to 48-hour incubation with simvastatin on the phosphorylation of Akt, we did demonstrate that a 24- to 48-hour preincubation with simvastatin inhibited VEGF-stimulated phosphorylation of Akt (Figure 7D). This effect was dose dependent and was observed at concentrations of simvastatin similar to those that inhibited VEGF-stimulated phosphorylation of flk-1/KDR and tube formation by HMECs cultured on 3D collagen gels. Simvastatin had no effect on the level of Akt expression.
localization of RhoA, these studies support the conclusion that HMG-CoA reductase inhibitors interfered with angiogenesis by inhibiting geranylgeranylation and membrane localization of RhoA. Given the dependence of angiogenesis on RhoA demonstrated in these studies, experiments also established that three signaling pathways, VEGF-stimulated phosphorylation of flk-1/KDR, Akt, and FAK, which have been shown to be dependent on Rho and to play a role in angiogenesis,8–10 are inhibited by HMG-CoA reductase inhibitors at concentrations similar to those that interfered with tube formation by HDMECs. The finding that angiogenesis is regulated by RhoA, taken together with the fact that the geranylgeranylation and membrane localization of RhoA is dependent on products of the cholesterol metabolic pathway, accounts for the relationship between cholesterol metabolism and angiogenesis demonstrated in these studies.

The finding that VEGF-stimulated angiogenesis in the CAM, FGF-stimulated angiogenesis in the corneal pocket, and tube formation by HDMECs in response to a cocktail of FGF, PMA, and VEGF were inhibited by simvastatin suggested that HMG-CoA reductase inhibitors might interfere with a common pathway or pathways downstream from these different proangiogenic stimuli. The finding that prolonged simvastatin treatment interfered with VEGF-stimulated activation of FAK and Akt at concentrations similar to those found to inhibit both tube formation by HDMECs and membrane localization of RhoA in HDMECs suggested that simvastatin inhibition of growth factor-stimulated Akt and FAK activation might play a role in the inhibition of angiogenesis by HMG-CoA reductase inhibitors.

The clinical relevance of these effects of HMG-CoA reductase inhibitors is supported by the observation that serum levels of simvastatin, which have been reported to be between 0.02 and 0.27 μmol/L in patients on therapeutic doses of these agents, are similar to those shown to inhibit both tube formation and membrane localization in these studies.16 The significance of the doses of simvastatin found to inhibit angiogenesis in the CAM and corneal pocket assays is complicated by the fact that in these assays, both the growth factors and simvastatin are delivered to the tissues via diffusion from a pellet or a mesh, respectively. Hence, the concentration of simvastatin may be higher in the immediate vicinity of the pellet and lower in the periphery of the pellet and in the tissue, and the actual effective concentration cannot be determined. However, the finding that simvastatin completely inhibited new blood vessel formation in the CAM and the cornea but had no effect on the underlying vasculature rules out a nonspecific toxic effect on preexisting capillaries.

Recently, several studies have suggested that HMG-CoA reductase inhibitors might stimulate angiogenesis. One study reported that a brief (1- to 5-hour) exposure of HUVECs expressing a DN-Akt mutant to HMG-CoA reductase inhibitors stimulated the formation of capillary-like structures on Matrigel, that brief exposure of HUVECs to HMG-CoA reductase inhibitors stimulated the phosphorylation of Akt, and that HMG-CoA reductase inhibitors stimulated collateral blood flow in the hind limbs of rabbits in which the femoral artery had been ligated.17 Finally, HMG-CoA reductase inhibitors have been reported to stimulate the migration and proliferation of bone marrow–derived endothelial progenitor cells.18,19

The apparent discrepancy between these findings and the present study may reflect differences in time course, cell type, and the current confusion in the literature regarding differences between angiogenesis and arteriogenesis. Because patients are treated for months and years with HMG-CoA reductase inhibitors, the effects of brief exposure of HUVECs to HMG-CoA reductase inhibitors may not be clinically significant. The antiangiogenic effect of HMG-CoA reductase inhibitors reported in the present study followed an 18-hour to 5-day incubation with the HMG-CoA reductase inhibitor and demonstrated that although prolonged incubation with simvastatin did not stimulate Akt phosphorylation, it did interfere with VEGF-stimulated Akt phosphorylation. Furthermore, unlike undifferentiated bone marrow endothelial cells, cell division and migration of the peripherally derived differentiated endothelial cells studied here have previously been shown to be inhibited by HMG-CoA reductase inhibitors.20 Finally, angiogenesis is a process in which endothelial cells proliferate and differentiate to form a fine capillary tree, which has not been shown to be correlated with increased blood flow, whereas arteriogenesis and the formation of collateral vessels in the rabbit hindlimb is a process in which endothelial cells and smooth muscle cells proliferate and develop muscular resistance vessels from preexisting arteriolar connections.21,22 Thus, data supporting the proangiogenic effects of HMG-CoA reductase inhibitors might reflect differences in cell type and time course and differences between models of angiogenesis and arteriogenesis.

Several recent studies support our conclusion that HMG-CoA reductase inhibitors interfere with angiogenesis. It has been reported that mice treated systemically with a combination of lovastatin and tumor necrosis factor-α demonstrate decreased tumor growth and capillary vessel formation in a Ras-transformed tumor model.23 Cerivastatin has been shown to inhibit migration and blood vessel formation in a transformed endothelial cell line.20 Furthermore, it has previously been reported that the number of blood vessels in the atherosclerotic lesions of cholesterol-fed monkeys decreases in animals treated with pravastatin compared with control animals,24 that systemically administered simvastatin interferes with new blood vessel formation by vasa vasorum in the coronary arteries of cholesterol-fed pigs,25 and that lesions in the retinas of patients with diabetic retinopathy treated with pravastatin also regress.26

Our data establish a specific effect of HMG-CoA reductase inhibitors on growth factor–stimulated new blood vessel formation in vivo. Furthermore, at concentrations that do not induce apoptosis, simvastatin is a potent inhibitor of tube formation by HDMECs. This antiangiogenic effect is dependent on the interference of HMG-CoA reductase inhibitors with the geranylgeranylation and the membrane localization of RhoA. Given the ever-increasing evidence of the involvement of angiogenesis in the pathogenesis of tumors, rheumatoid arthritis, psoriasis, diabetic retinopathy, and atherosclerosis,1 HMG-CoA reductase inhibitors could play an important role in the treatment and prevention of these diseases.
Note Added in Proof
After submission of this manuscript, other studies (Weis et al. and Urbich et al.) reported that atorvastatin and cerivastatin have an antiangiogenic effect at concentrations similar to those shown by the present study to interfere with tube formation.

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