Statins Augment Collateral Growth in Response to Ischemia but They Do Not Promote Cancer and Atherosclerosis

Masataka Sata, Hiroaki Nishimatsu, Jun-ichi Osuga, Kimie Tanaka, Nobukazu Ishizaka, Shun Ishibashi, Yasunobu Hirata, Ryozo Nagai

Abstract—3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, or statins, are widely prescribed to lower cholesterol. Recent reports suggest that statins may promote angiogenesis in ischemic tissues. It remains to be elucidated whether statins potentially enhance unfavorable angiogenesis associated with tumor and atherosclerosis. Here, we induced hind limb ischemia in wild-type mice by resecting the right femoral artery and subsequently inoculated cancer cells in the same animal. Cerivastatin enhanced blood flow recovery in the ischemic hind limb as determined by laser Doppler imaging, whereas tumor growth was significantly retarded. Cerivastatin did not affect capillary density in tumors. Cerivastatin, pitavastatin, and fluvastatin inhibited atherosclerotic lesion progression in apolipoprotein E-deficient mice, whereas they augmented blood flow recovery and capillary formation in ischemic hind limb. Low-dose statins were more effective than high-dose statins in both augmentation of collateral flow recovery and inhibition of atherosclerosis. These results suggest that statins may not promote the development of cancer and atherosclerosis at the doses that augment collateral flow growth in ischemic tissues. (Hypertension. 2004;43:1214-1220.)

Key Words: cholesterol ▪ atherosclerosis ▪ nitric oxide ▪ circulation

A
ngiogenesis is a physiological response to ischemia. In animal models of ischemia, a large body of evidence indicates that administration of angiogenic growth factors can augment nutrients perfusion through neovascularization.1 Therapeutic angiogenesis, a strategy to cure tissue ischemia by promoting the proliferation of collateral vessels, has emerged as one of the most promising therapies developed to date.2 Early clinical trials reported that the administration of angiogenic growth factors as a recombinant protein or gene might enhance the formation of new collateral vessels, relieving some ischemic symptoms.1 However, the strategy is associated with the dilemma that these angiogenic substances could promote unfavorable angiogenesis associated with tumors, diabetic retinopathy, and atherosclerosis.2,3

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, or statins, are widely used to lower cholesterol levels. Large trials have demonstrated that statins reduce the mortality and the incidence of cardiovascular events.4 Statins possess lipid-independent benefits, including improvement of endothelial function, inhibition of inflammation, and reduction of myocardial or cerebral infarction size.5 It is reported that statins promote angiogenesis in response to ischemia in normocholesterolemic animals.6,7 Although these studies raised clinical enthusiasm because statins could be used for therapeutic angiogenesis, there remains a concern that statins may promote tumors, diabetic retinopathy, and atherosclerosis by stimulating neovascularization.8 Therefore, our studies were designed to examine the effects of stains on physiological and pathological angiogenesis in the same animal.

Methods

Mouse Hind Limb Ischemia Model

Wild-type C57BL/6J mice were purchased from SLC Japan (Shizuoka, Japan). Apolipoprotein E-deficient (ApoE−/−) mice were purchased from Jackson Laboratory (Bar Harbor, Me). Unilateral hind limb ischemia was induced, and hind limb blood perfusion was measured as described.7 Saline or cerivastatin (6 mg/kg per day) was administered subcutaneously every day starting 3 days before surgery. Vehicle (0.5% carboxymethyl cellulose), pitavastatin (1 or 10 mg/kg per day), or fluvastatin (5 or 25 mg/kg per day) was administered every day by gavage. At 5 weeks, microangiography was performed using barium sulfate as a contrast medium.8 Images were acquired by a digital x-ray transducer system (DSR-1000AD, Hitachi, Tokyo). The thigh muscles were harvested at the time point indicated and stained for von Willebrand factor (DAKO, Kyoto) or CD31 (PharMingen, San Diego, Calif) to detect endothelial cells.7

Tumor Implantation Model

The 2×10⁶ murine syngeneic colon cancer cells (CMT93, American Type Culture Collection, Rockville, Md) were inoculated into the left flank fold of C57BL/6J mice, whose right femoral arteries had been excised on the same day.9 Blood perfusion of the tumor was assessed...
using an LDPI system and expressed as the ratio of perfusion in the tumor versus that in the navel. At 5 weeks, tumors were excised and fixed in methanol. Capillaries were identified by positive staining for CD31 and morphology.7

**Cell Proliferation Assay In Vitro**

CMT93 cells were maintained in Dulbecco modified eagle medium and synchronized in 0.1% fetal bovine serum (FBS) for 72 hours. Cells were then stimulated to proliferate for 36 hours with 10% FBS in the presence of cerivastatin as indicated. DNA content was analyzed by flow cytometry (EPICS XL; Beckman Coulter, Fullerton).10 MTS assay was performed as described.11

**Analysis of Atherosclerotic Lesions**

ApoE−/− mice were fed a western-type diet (0.15% cholesterol, 15% butter) for 5 weeks after induction of hind limb ischemia. Lipid deposition was quantified by en face aorta analysis as previously described.12 The effect of statins on endothelium-dependent vasodilatation of atherosclerotic lesions was evaluated by relaxation of aortic rings in response to acetylcholine.7 Eighteen-week-old wild-type or ApoE−/− mice were treated with either saline or cerivastatin (6 mg/kg per day, subcutaneous) for 7 days, and the thoracic aortas were excised. Relaxation of the aortic rings in response to acetylcholine was monitored.7

**Statistics**

All data are expressed as the mean value±SEM. Statistical comparisons of means were performed by ANOVA followed by Student t test. P<0.05 was considered to be statistically significant.

**Results**

**Effects of Cerivastatin on Collateral Development and Tumor Growth**

Murine syngeneic colon cancer cells were inoculated into the left flank fold of 30- to 35-week-old male wild-type C57BL/6 mice, in which hind limb ischemia had been induced by resecting the right femoral artery. Cerivastatin significantly augmented blood flow recovery (Figure 1A). The capillary density of the ischemic leg was increased by cerivastatin (saline, 711±42/mm²; cerivastatin, 808±56/mm²), but the difference did not reach statistical significance (Figure 1B). A tumor developed in the subcutaneous space where CMT93 cancer cells had been inoculated (Figure 1C). In the mice treated with cerivastatin, the tumor was significantly smaller than that in the mice treated with saline (saline, 153±82 mm³; cerivastatin, 31±16 mm³). Laser Doppler imaging revealed...
an increase in blood flow around the tumor in both groups (Figure 1D). Cerivastatin did not significantly affect blood flow (ratio: saline, 2.2±0.4; cerivastatin, 2.2±0.6) or capillary density in the tumor (saline, 1339±263/mm²; cerivastatin, 1108±93/mm²) (Figure 1E).

To elucidate the mechanism by which cerivastatin inhibits tumor growth without affecting tumor-associated angiogenesis, direct effect of statins on cancer cell proliferation was investigated in vitro. When the cancer cells were stimulated to proliferate in the presence of high serum, DNA content analysis revealed that cerivastatin decreased the number of cells in S or G2/M phase (control, G1: 44.6%, S: 3.6%, G2/M: 23.4%) (Figure 3F). Cerivastatin also inhibited proliferation of CMT93 cells in a dose-dependent manner as determined by total cell number (Figure 2B) and MTS assay (Figure 2C). These results suggest that direct inhibitory effects of statins on cell proliferation may mediate, at least in part, their antitumor effects.

Effects of Statins on Collateral Growth and Atherosclerotic Lesion Progression in Hyperlipidemic Mice

Eight-week-old ApoE−/− male mice were fed a western-type diet and treated with saline or cerivastatin. After 1 week, we generated hind limb ischemia in the mice. Cerivastatin significantly enhanced recovery of blood flow after acute ischemia (Figure 3A). Although there was no significant difference in the number of angiographically visible collateral vessels at 5 weeks (Figure 3B), anti-CD31 immunostaining revealed that cerivastatin significantly increased the density of histologically detectable capillaries in the ischemic leg (saline, 464±68/mm²; cerivastatin, 630±287/mm²) (Figure 3C). Consistent with previous reports,13 there was no significant difference in the lipid profile between the mice treated with saline and those treated with cerivastatin (total cholesterol, 630±64 versus 470±53 mg/dL; triglycerides, 84±17 versus 51±11 mg/dL; HDL cholesterol, 15±2 versus 11±1 mg/dL). However, cerivastatin markedly inhibited atherosclerotic progression (Figure 3D). The number of vessels in atherosclerotic lesions at the aortic root (Figure 3E) was significantly smaller in the mice treated with cerivastatin than that in the mice treated with saline (saline, 44.6±7.0/mm²; cerivastatin, 31.3±3.5/mm²) (Figure 3F).

Nine-week-old male ApoE−/− mice were treated with vehicle or pitavastatin (1 mg/kg per day or 10 mg/kg per day) every day by gavage and fed a western-type diet. After 3 weeks, hind limb ischemia was generated. There was no significant difference in the lipid profile among the mice treated with vehicle or pitavastatin (Table). Low-dose pitavastatin (1 mg/kg per day), but not high-dose pitavastatin (10 mg/kg per day), significantly accelerated recovery of blood flow in the ischemic hind limb (Figure 4A). Histological examination revealed that low-dose pitavastatin increased capillary density in the ischemic muscle at 5 weeks (Figure 4B). En face aorta analysis revealed that pitavastatin significantly inhibited atherosclerotic lesion progression (Figure 4C). Interestingly, beneficial effects of pitavastatin on both collateral growth and atherosclerosis were attenuated at a higher dose.

Dose-dependent effect of statins on ischemia-induced collateral formation was also investigated by oral administration of fluvastatin, one of the widely prescribed statins. Adult male 24- to 36-week-old C57/BL6J mice were treated with vehicle or fluvastatin (5 mg/kg per day or 20 mg/kg per day) every day by gavage. After 3 weeks, hind limb ischemia was generated. Histological examination revealed that fluvastatin increased capillary density in the ischemic muscle at 5 weeks (Figure 5A). Consistent with the findings with pitavastatin, lower-dose fluvastatin (5 mg/kg per day) was more effective than high-dose fluvastatin (20 mg/kg per day) in promoting neovascularization. Next, atheroprotective effect of low-dose
fluvastatin was evaluated in 8-week-old female ApoE<sup>-/-</sup> mice. After 16 weeks, en face aorta analysis revealed that low-dose fluvastatin significantly inhibited atherosclerotic lesion formation (Figure 5B). Taken together, these results suggest that statins can inhibit atherosclerotic lesion formation at low doses that promote ischemia-induced collateral vessel growth.

Effect of Statins on Endothelium-Dependent Relaxation of Atherosclerotic Lesions

To obtain insights into the mechanism by which statins augment collateral growth in response to ischemia without accelerating the development of cancer and atherosclerosis, we evaluated the effect of statins on endothelium-dependent vasodilatation of atherosclerotic lesions (Figure 6). Compared with the aortas taken from age-matched wild-type C57BL/6 mice, endothelium-dependent vasorelaxation was markedly impaired in aortas of ApoE<sup>-/-</sup> mice treated with saline.

**Serum Lipid Profile of ApoE<sup>-/-</sup> Mice Treated With Vehicle or Pitavastatin (mg/dL)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>T. Chol</th>
<th>Triglycerides</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (0.5% carboxymethylcellulose)</td>
<td>666±218</td>
<td>66±16</td>
<td>43±15</td>
</tr>
<tr>
<td>Pitavastatin (1 mg/kg per day)</td>
<td>713±163</td>
<td>55±7</td>
<td>15±4</td>
</tr>
<tr>
<td>Pitavastatin (10 mg/kg per day)</td>
<td>876±112</td>
<td>53±14</td>
<td>16±3</td>
</tr>
</tbody>
</table>

* T. Chol indicates total cholesterol; HDL, high-density lipoprotein cholesterol.
Cerivastatin treatment partially restored the endothelium-dependent relaxation of atherosclerotic aortas.

**Discussion**

Recent reports that statins enhance ischemia-induced angiogenesis\(^6^,^7^,^14^,^15\) raised clinical concerns that statins may potentially promote tumor progression by enhancing angiogenesis.\(^16\) However, clinical studies reported no association between long-term treatment with statins and the risk of cancers.\(^17\) Conversely, experimental studies reported that statins significantly reduced tumor growth with a reduction in tumor vascularization in Lewis lung cancer model.\(^18^,^19\) Consistently, a randomized controlled trial demonstrated that pravastatin prolonged the survival of patients with advanced hepatocellular carcinoma at 40 mg/d,\(^20\) which is a standard dose for lipid-lowering therapy. In this study, cerivastatin (6 mg/kg per day) inhibited tumor growth in a colon cancer model, whereas collateral flow development in the ischemic hind limb was augmented in the same animal. Cerivastatin did not affect average blood flow or capillary density in tumor. Cerivastatin inhibited proliferation of CMT-93 cells in vitro in a dose-dependent manner. Most likely, the antitumor effect of statins was mediated, at least in part, by direct effects of statins on colon cancer cells including inhibition of proliferation, induction of apoptosis and inhibition of invasiveness,\(^19\) which are independent of tumor-induced angiogenesis.

There is a large body of clinical evidence that statin therapy suppresses atherosclerotic lesion progression, even in patients with normal cholesterol level.\(^4\) In this study, statins inhibited atherosclerotic lesion progression without significantly affecting circulating cholesterol level. Neovascularization in atheroma was significantly inhibited, whereas collateral vessel development was enhanced by statins in the ischemic muscles of the same animal. Statin therapy partially restored impaired endothelial function of atherosclerotic vessel wall. Most likely, statins inhibit atherosclerotic lesion development by their pleiotropic effects.\(^4\) The decrease in capillary density in atheroma appears to be secondary to inhibition of lesion formation by statin therapy.

To explain the puzzling effects of statins on physiological and pathological angiogenesis, it was proposed that statins have a biphasic dose-dependent effect on angiogenesis, ie, proangiogenic at low therapeutic doses (0.5 mg/kg per day of cerivastatin) but angiostatic at high doses (2.5 mg/kg per day),\(^18\) based on their observations in mouse models of inflammation and tumor-induced angiogenesis. In this study, cerivastatin augmented collateral vessel growth in response to...
Acute ischemia at even a higher dose (6 mg/kg per day), ≈1000-fold that for human use. It might be plausible that proangiogenic or antiangiogenic effects of statins might also depend on distinct mechanisms of angiogenesis associated with cancer, tissue ischemia, or inflammation. Statins probably function to promote collateral vessel growth only in ischemic tissues without having significant proangiogenic effects in atherosclerosis, tumor, and diabetic retinopathy. Statins may inhibit the development of atherosclerosis and cancer through their pleiotropic effects. Consistent with this notion, a low dose (1 mg/kg per day) of pitavastatin was more effective than a high dose (10 mg/kg per day) in increasing blood flow to ischemic tissue and inhibiting atherosclerotic lesion formation.

Although cerivastatin significantly augmented recovery of blood flow in both wild-type mice and ApoE<sup>−/−</sup> mice, we detected significant increase in capillary density only in ApoE<sup>−/−</sup> mice. It is likely that statins may be more effective in hyperlipidemic mice with impaired endothelial function than in normocholesterolemic mice.

**Perspectives**

Our findings suggest that statins may not promote cancer and atherosclerosis by stimulating pathological angiogenesis at doses that increase collateral blood flow in ischemic tissue. Statin therapy may be advantageous in patients with ischemic diseases, although it remains to be determined whether statins can achieve angiogenic effects as potently as conventional growth factors in patients.

**Acknowledgment**

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