Simvastatin Preserves the Structure of Coronary Adventitial Vasa Vasorum in Experimental Hypercholesterolemia Independent of Lipid Lowering

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Background—Previous studies have demonstrated that experimental hypercholesterolemia leads to neovascularization in the coronary artery vasa vasorum (VV). Recent evidence suggests that HMG-CoA reductase inhibitors (statins) have beneficial effects independent of lipid lowering. We aimed to determine the effect of simvastatin on coronary VV neovascularization, in the absence of cholesterol lowering.

Methods and Results—Pigs were randomized to 3 groups fed a normal (N), high cholesterol (HC), or HC+simvastatin (HC+S) diet for 12 weeks. The proximal left anterior descending artery was isolated, scanned with micro-CT, and reconstructed. Quantification of the VV density in serial cross-sections along the vessel was then performed. LDL cholesterol was similarly increased in HC and HC+S compared with N. There was an increase in both VV density (4.7±0.3 versus 2.7±0.2 n/mm²; P<0.05) and vessel wall area (3.1±0.2 versus 1.8±0.1 mm²; P<0.05) in HC compared with N. The VV density in HC+S was preserved compared with HC (3.0±0.2 n/mm²; P<0.05), despite similar increase in vessel wall area compared with N (2.5±0.1 mm²; P<0.05). Coronary artery tissue expression of VEGF was increased in HC but not in HC+S compared with N. In parallel, immunoreactivity for HIF-1α, VEGF, MMP-2, and MMP-9 was accentuated in the outer media in HC but not in HC+S compared with N.

Conclusions—This study demonstrates that simvastatin attenuates hypoxia in the coronary artery wall and VV neovascularization in experimental hypercholesterolemia, despite no change in plasma lipids. These data are consistent with an additional mechanism for the vascular effects of the statins, independent of cholesterol lowering. (Circulation. 2002;105:415-418.)

Key Words: hypercholesterolemia ■ pathological neovascularization ■ statins

The vasa vasorum (VV) are blood vessels present in the adventitial layer of the vessel wall. They presumably exist to nourish large blood vessels, including the epicardial coronary arteries. Previous studies have demonstrated a correlation between extent of VV neovascularization and severity of coronary atherosclerosis. Moreover, we have previously demonstrated that VV neovascularization occurs before the development of atherosclerotic plaque. One potential mechanism might be endothelial dysfunction of the VV at an early stage, leading to reduction in oxygen supply to the coronary artery wall and subsequent activation of hypoxia-inducible factor (HIF), a transcription factor that regulates the expression of proangiogenic factors such as vascular endothelial growth factor (VEGF).3,4

Treatment of hyperlipidemia with HMG-CoA reductase inhibitors (statins) reduces coronary events in humans.5,6 In addition, recent evidence further suggests that statin drugs may have important vasculoprotective effects, such as improvement of endothelial function of both the microcirculatory and the macrocirculatory bed, independent of lipid lowering.7–9 The present study was designed to test the hypothesis that experimental hypercholesterolemia is associated with the induction of HIF as well as proangiogenic factors, including VEGF and matrix metalloproteinases (MMPs), in the coronary arterial wall. Furthermore, we hypothesized that simvastatin attenuates the expression of these factors as well as coronary VV neovascularization in the absence of cholesterol lowering.

Methods

Animals
The study was approved by the Institutional Animal Care and Use Committee. Experiments were conducted on female crossbred pigs (25 to 37 kg) (Larson Products, Sargeant, Minn),9–11 randomized to

Received October 29, 2001; revision received December 4, 2001; accepted December 19, 2001.
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either a normal diet (N, n=5) or a high cholesterol diet (TD 932956, Harlan Teklad) without (HC, n=5) or with 40 to 80 mg of simvastatin per day (HC+S, n=4) for 12 weeks. At the end of the study period, blood samples were taken for lipid parameter analysis, and the heart and coronary arteries were harvested immediately after euthanasia.\textsuperscript{12}

### Micro–Computed Tomography (Micro-CT) Analysis

Segments of left anterior descending coronary artery (2 cm) were prepared and scanned by micro-CT in standardized, blinded manner.\textsuperscript{13,14} This yielded a picture with a 3D matrix of 42-μm cubic voxels with 16 bits of gray scale. Data analysis was performed blinded to treatment using Analyze software (Biomedical Imaging Resource).\textsuperscript{3} On average, 6 to 12 cross-sections at 1-mm intervals, in areas between branch points, were chosen for region of interest analysis. The area of VV analysis was determined as previously described and designated vessel wall area.\textsuperscript{3,14} VV were manually traced and measured in this area on each cross-section, yielding the following parameters: vessel wall area, VV count, and VV density (ie, VV/mm\textsuperscript{2} vessel wall area), mean diameter of 1st- and 2nd-order VV, and ratio of 2nd- to 1st-order VV. First-order VV originated from the coronary lumen and ran longitudinally, 2nd-order VV originated from 1st-order VV and ran circumferentially.\textsuperscript{3}

### Western Blotting

Equal amounts of protein from whole artery lysates from 3 pigs in each experimental group were resolved on a 10% SDS-polyacrylamide gel. Ponceau staining was used to ensure equal protein loading. Immunoblotting was performed using a polyclonal rabbit antibody to VEGF (Santa-Cruz, Calif) followed by a secondary anti-rabbit antibody conjugated to horseradish peroxidase (Amer- sham, Ill).

### Immunohistochemistry

After deparaffinization and rehydration, coronary artery slides were incubated with 3% H\textsubscript{2}O\textsubscript{2}/100% MeOH to block endogenous tissue peroxidase activity.\textsuperscript{9} Primary antibodies (anti-VEGF, dilution 1:100; anti-HIF 1α, Novus Biologicals, dilution 1:50; anti-precursor and active) MMP-2 and MMP-9, Chemicon International, dilution 1:200) were applied at 4°C overnight and detected with the EnVision kit (Dako Corp) and 3,3-diaminobenzidine tetrahydrochloride as chromogen (Vector Laboratories, Inc). Incubation with an unspecific isotype antibody served as a control for the specificity of immunoreactivity. All sections were counterstained with hematoxylin.

### Statistical Analysis

Continuous data are expressed as mean±standard error. Group comparison of parametric and nonparametric data were made by t and U test, respectively. Multiple group comparison was based on analysis of variance (ANOVA) followed by post hoc analysis. Statistical significance was accepted for a value of \( P<0.05 \).

### Results

#### Plasma Lipid Parameters

Compared with pigs fed a normal diet, plasma lipid concentrations were increased in HC and HC+S pigs (total cholesterol: 84±2, 619±122, 565±161 mg/dL, \( P<0.01 \); LDL cholesterol: 36±1, 495±109, 416±134 mg/dL, \( P<0.05 \); HDL cholesterol: 41±3, 65±20, 136±29 mg/dL, \( P<0.05 \)).

#### Vasa Vasorum

Compared with the N group, VV density and vessel wall area were increased in the HC group but not in the HC+S group (Table). The architecture of VV in N animals was clearly structured into 1st- and 2nd-order VV (Figure 1A). In the HC group, a plexus of newly formed VV was apparent, in association with an increased ratio of 2nd- to 1st-order VV (\( P<0.001 \) HC compared with N), which was not found in HC+S. In addition, diameter of the 1st-order VV was higher in the HC group compared with both the N (\( P=0.01 \)) and HC+S group (\( P=0.02 \)).

#### Western Blotting for VEGF

VEGF protein content in the vessel wall was higher in the HC than in the N group. This was preserved in the HC+S group (Figure 1B).

### Immunoreactivity for HIF-1α, VEGF, MMP-2, and MMP-9

Intense immunoreactivity for hypoxia and angiogenesis factors, including MMP-2 and MMP-9, was seen in HC only and primarily in the outer smooth muscle cell layers of the media. In addition, endothelial cells of VV stained strongly positive for VEGF in HC (Figure 2).

### Discussion

This study demonstrates that simvastatin attenuates the increase in coronary VV density in the absence of any lipid lowering effect in experimental HC. These changes occurred in association with an attenuation of the expression of HIF-1α, VEGF, MMP-2, and MMP-9, suggesting attenuation of local vessel wall hypoxia and compensatory neovascularization by a direct, non–lipid-lowering effect of simvastatin.

Our data suggest that simvastatin inhibits coronary VV neovascularization in vivo in early atherosclerosis. Previous data are conflicting regarding the effect of statins on neovascularization. Recent data suggests that simvastatin activates the protein kinase Akt and promotes blood vessel formation in ischemic limbs.\textsuperscript{15} In contrast, other studies have suggested that pravastatin decreases neovascularization in advanced atherosclerosis.\textsuperscript{16} Furthermore, recent studies demonstrated that statins suppress the production of VEGF as well as...
MMPs in vivo and in vitro.\textsuperscript{17,18} This may result from a differential effect of statins in varying pathophysiological conditions. The changes noted in our study occurred without an alteration in cholesterol levels, implying a direct effect of statins on VV growth.

We have previously demonstrated that coronary adventitial VV neovascularization in experimental HC occurred before development of atherosclerotic plaque.\textsuperscript{3} This process may reflect a response to local hypoxia in the vessel wall, with release of growth factors, such as VEGF. Indeed, expression of HIF-1\textalpha was found in the coronary artery wall in HC, which was not seen in animals on both high cholesterol diet and simvastatin. One potential mechanism, explaining these findings, is a dysfunctional state of the endothelium of VV in HC and its attenuation by simvastatin. This hypothesis is supported by multiple studies demonstrating that statins improve coronary endothelial function.\textsuperscript{19} Moreover, we have recently reported that simvastatin preserved coronary microvessel endothelial function in experimental HC without effecting cholesterol levels.\textsuperscript{8} In addition, a decrease in basal VV tone may play a role in the apparent increase in diameter that occurred in both 1st- and 2nd-order VV following simvastatin treatment.

In vitro studies are further elucidating the cellular mechanisms for the non–lipid-lowering effects of HMG-CoA reductase inhibitors. Apart from limiting cholesterol synthesis, statins inhibit the synthesis of isoprenoids such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which are essential for membrane translocation and biological activity of GTPase family members such as Ras and RhoA, respec-

**Figure 1.** A, Representative micro-CT images of coronary arteries from normal (N, left), high cholesterol (HC, middle), and high cholesterol+simvastatin (HC+S, right) groups. Note the dense plexus of microvessels in the HC but not in the HC+S group (reconstruction voxel size: 21 \(\mu\)m). B, Immunoblots of porcine coronary tissue homogenates (50 \(\mu\)g protein/lane) of 3 normal (N), 3 hypercholesterolemic (HC), and 3 high cholesterol+simvastatin (HC+S) animals for VEGF protein. Mean densitometry of the 3 groups is shown below. *\(P<0.05\) N and HC+S compared with HC.

Acknowledgments

This work was supported by grants from the National Institutes of Health (R01 HL63911, R01 HL63282, and R01 HL65342), the Miami Heart Research Institute, the Mayo Foundation, the Rappaport Program in Vascular Biology, and a Merck Lipid Medical School Unrestricted Grant.

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Circulation. 2002;105:415-418
doi: 10.1161/hc0402.104119
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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