Altered Myocardial Microvascular 3D Architecture in Experimental Hypercholesterolemia

Martin Rodriguez-Porcel, MD; Amir Lerman, MD; Erik L. Ritman, MD, PhD; Stephanie H. Wilson, MD; Patricia J.M. Best, MD; Lilach O. Lerman, MD, PhD

Background—Experimental hypercholesterolemia (HC) impairs intramyocardial microvascular function. However, whether this is associated with alterations in microvascular architecture remained unknown. Using a novel 3D micro-CT scanner, we tested the hypothesis that HC is associated with an alteration in the microvascular architecture.

Methods and Results—Pigs were euthanized after 12 weeks of either normal (n=6) or 2% HC (n=6) diet. The hearts were excised and the coronary arteries injected with a radiopaque contrast material. Myocardial samples were scanned with micro-CT, and 3D images were reconstructed with 21-µm cubic voxels. The myocardium was tomographically subdivided into subepicardium and subendocardium, and microvessels (<500 µm in diameter) were counted in situ within each region. In the subendocardium of HC pigs, the intramyocardial density of microvessels was significantly higher than in normal animals (1221.4±199.7 versus 758.3±90.8 vessels/cm³, P<0.05) because of an increase in the number of microvessels <200 µm in diameter (1214.4±199.7 versus 746.6±101.5 vessels/cm³, P<0.05). The subepicardial vascular density was similar in both groups.

Conclusions—HC has differential effects on the spatial density of the subendocardial microvasculature that may play a role in regulation and/or spatial distribution of myocardial blood flow. This study also demonstrates the feasibility of studying myocardial microvascular architecture with micro-CT in pathophysiological states. (Circulation. 2000;102:2028-2030.)

Key Words: hypercholesterolemia ▪ tomography ▪ microcirculation

Hypercholesterolemia (HC) is a major risk factor for coronary artery disease. HC is used in experimental animals as a surrogate for early coronary atherosclerosis and is characterized by impaired endothelial function of the epicardial coronary arteries. Even in the absence of overt atherosclerotic changes in the vascular wall, these functional abnormalities are associated with subtle morphological alterations, such as an increase in the density of vasa vasorum in the coronary adventitia.

Like the epicardial arteries, the intramyocardial microvessels (diameter <500 µm) may also manifest endothelial dysfunction due to HC and may conceivably undergo alterations similar to those observed in the vasa vasorum microvessels. However, whether the microvascular functional impairment in experimental HC is accompanied by alterations in architecture remains unknown, largely because of the lack of accurate and high-resolution methods to study the intact 3D myocardial microvascular architecture.

Micro computed tomography (micro-CT) is a novel and powerful technique that permits assessment of the 3D pattern of microvascular structure and provides a useful means for studying the spatial distribution of vessels within an organ. This technique may therefore provide a unique insight into the early structural changes of the intramyocardial vascular tree in HC. Thus, the present study was designed to test the hypothesis that HC would be associated with alterations in the density pattern of the intramyocardial microvascular tree.

Methods

All procedures were approved by the Institutional Animal Care and Use Committee. Female domestic pigs (23 to 35 kg) were fed for 12 weeks with either a normal (n=6) or atherogenic (n=6) diet of 2% cholesterol and 15% lard (TD-93296, Harlan Teklad). Blood samples were collected after completion of the diet for measurement of plasma cholesterol levels, and the animals were then euthanized with intravenous pentobarbital (20 mL of Sleepaway [Fort Dodge Laboratories]).

The heart was removed, and the distal left anterior descending coronary artery was cannulated and perfused with a solution of 0.9% normal saline and heparin at flow rate of 10 mL/min. When the perfusate draining through the veins was free of blood, radiopaque Microfil silicone rubber (MV-122, Flow Tech, Inc) was perfused through the cannulated left anterior descending coronary artery at a flow rate of 0.9 mL/min (physiological perfusion pressure of 80 mm Hg). This contrast agent essentially remains in the intravascular compartment. Filling was deemed complete when the Microfil flowed freely from the myocardial veins, and the hearts were kept at 4°C for 1 day. A transmural portion of the left ventricular myocardium (~2×1×1 cm) was then sectioned and prepared as previously described.

Circulation is available at http://www.circulationaha.org
coronary vascular resistance, and large microvessels (diameters between 200 and 500 μm) were considered subendocardium.

The micro-CT scanner has been described previously. Myocardial samples were scanned at 0.49° angular increments, providing 721 views around 360°. Images were recorded, digitized, and transferred to a controlling computer. The 3D volume images, reconstructed with a modified Feldkamp’s filtered backprojection algorithm, consisted of cubic voxels of 21 μm on a side and were displayed at 41 μm resolution. The radiopacity of each voxel was represented by a 16-bit gray-scale value.

Image analysis was performed with the Analyze software package (Biomedical Imaging Resource, Mayo Foundation), which provides methods to compute, display, and analyze sections from reconstructed volume images. The myocardium was divided into 3 equal parts, and data were analyzed in slices obtained at equal intervals from each third. In line with coronary artery distribution and regulation, the outer two thirds of the myocardium were considered subepicardium, and the inner one third was considered subendocardium.

In each region, microvessels (diameters <500 μm) were counted. Microvessels were further classified as small microvessels (diameters between 82 and 199 μm), which are responsible for most of the coronary vascular resistance, and large microvessels (diameters between 200 and 500 μm), whose diameters were also measured.

In addition, by use of a "connectivity" software that allows for isolation of a vessel, a single epicardial artery and its branches were tomographically dissected in each pig, and their branching pattern was visually assessed.

Statistical Analysis
Data are expressed as mean±SEM. Frequency distribution of large microvessels was calculated as the relative contribution (%) of each size category. Comparisons between the groups were performed with unpaired Student’s t test. Statistical significance was accepted for a value of P≤0.05.

Results
Compared with controls, HC pigs had significantly higher serum levels of total (272±41 versus 84±11 mg/dL, respectively, P<0.05) and LDL cholesterol, whereas triglyceride levels were similar. Figure 1 (top) illustrates representative 3D micro-CT images of the swine intramyocardial microvasculature, demonstrating a visually appreciable increase in intramyocardial microvascular density in the subendocardium of HC pigs. Figure 1 (bottom) depicts tomographically isolated coronary arteries, showing increased density of small branches (diameters 90 to 130 μm) in HC, mainly in the distal part of the vessel. The total number of subendocardial microvessels was significantly higher in HC than in normal pigs (Figure 2) because of increased numbers of small microvessels (Table). This was associated with a decreased number of large microvessels (mainly those with 200- to 299-μm diameters, Table), although their frequency distribution (data not shown) was similar to that of controls (P>0.05 for each category).

The numbers of large and small subepicardial microvessels were similar in both groups (Table). Mean diameters of large microvessels were similar in normal and HC pigs in both the subepicardium (270±20 and 280±20 μm, respectively, P=NS) and subendocardium (250±20 and 240±20 μm, respectively, P=NS).

Discussion
This study demonstrates that HC is associated with increased microvascular density and sprouting in the subendocardial

### Spatial Density of Small (82–199 μm) and Large (200–500 μm) Intramyocardial Microvessels in Normal and HC Pigs Measured With Micro-CT

<table>
<thead>
<tr>
<th>Microvessels</th>
<th>Normal</th>
<th>Hypercholesterolemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subepicardium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small (82–199 μm)</td>
<td>817.7±192.2</td>
<td>783.5±64.5</td>
</tr>
<tr>
<td>Large</td>
<td>10.4±2.3</td>
<td>8.0±1.6</td>
</tr>
<tr>
<td>200–299 μm</td>
<td>7.7±0.49</td>
<td>6.8±0.8</td>
</tr>
<tr>
<td>300–399 μm</td>
<td>1.5±0.2</td>
<td>1.2±0.4</td>
</tr>
<tr>
<td>400–499 μm</td>
<td>1.2±0.7</td>
<td>0.9±0.4</td>
</tr>
<tr>
<td>Subendocardium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small (82–199 μm)</td>
<td>746.6±101.5</td>
<td>1214.4±199.7*</td>
</tr>
<tr>
<td>Large</td>
<td>11.2±1.3</td>
<td>6.5±1.7*</td>
</tr>
<tr>
<td>200–299 μm</td>
<td>8.9±0.9</td>
<td>5.1±0.7*</td>
</tr>
<tr>
<td>300–399 μm</td>
<td>1.3±0.6</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>400–499 μm</td>
<td>0.8±0.5</td>
<td>0.0±0.0</td>
</tr>
</tbody>
</table>

Values are number per cm³. *P<0.05 vs. normal.
myocardium and may suggest myocardial neovascularization in HC. HC is characterized by impaired microvascular endothelial function, but its effect on myocardial microvascular architecture remained undefined. Formation of capillaries in limb muscle and of new vessels in rabbit aorta wall is not increased in HC. However, neovascularization has been shown around atherosclerotic lesions in the carotid artery and in the coronary adventitia in the absence of plaque formation. Our study demonstrates that early HC is associated with augmented vascularization of intramyocardial microvessels.

The mechanisms responsible for this phenomenon may be multifactorial. HC stimulates the release of growth factors, potentially leading to new vessel growth. The latter may also be a response to tissue hypoxia, because HC is associated with myocardial perfusion defects. Hypoxic tissue is a potential source of angiogenic factors, and episodes of inadequate oxygen supply in HC may conceivably stimulate new vessel formation. In our study, vascularization increased selectively in the subendocardium, possibly because this region autoregulates less effectively and has a greater oxygen consumption than the subepicardium and is thus more susceptible to ischemia. Our study also suggests that at least some of these new vessels may sprout from preexisting intramyocardial arterioles.

The overall increase in microvessels observed might be due primarily to an increase in small microvessels, which are largely derived from intramyocardial arterioles. The mechanisms responsible for this phenomenon may be multifactorial. HC stimulates the release of growth factors, potentially leading to new vessel growth. The latter may also be a response to tissue hypoxia, because HC is associated with myocardial perfusion defects. Hypoxic tissue is a potential source of angiogenic factors, and episodes of inadequate oxygen supply in HC may conceivably stimulate new vessel formation. In our study, vascularization increased selectively in the subendocardium, possibly because this region autoregulates less effectively and has a greater oxygen consumption than the subepicardium and is thus more susceptible to ischemia. Our study also suggests that at least some of these new vessels may sprout from preexisting intramyocardial arterioles.

The overall increase in microvessels observed might be due primarily to an increase in small microvessels, which are largely derived from intramyocardial arterioles. The mechanisms responsible for this phenomenon may be multifactorial. HC stimulates the release of growth factors, potentially leading to new vessel growth. The latter may also be a response to tissue hypoxia, because HC is associated with myocardial perfusion defects. Hypoxic tissue is a potential source of angiogenic factors, and episodes of inadequate oxygen supply in HC may conceivably stimulate new vessel formation. In our study, vascularization increased selectively in the subendocardium, possibly because this region autoregulates less effectively and has a greater oxygen consumption than the subepicardium and is thus more susceptible to ischemia. Our study also suggests that at least some of these new vessels may sprout from preexisting intramyocardial arterioles.

In summary, our study using a powerful and novel micro-CT technique describes, for the first time, the 3D microvascular structure in normal and HC pig myocardium. Our results show that HC selectively enhances microvascular density in the subendocardial myocardium. The vessel size and location specificity of the changes observed in our study may also partly explain the variable findings regarding the ability of HC to induce new vessel growth.

Acknowledgments
This study was funded by NIH grants HL-03621 and HL-63282 and a grant from the Bruce and Ruth Rappaport Program in Vascular Biology.

References
Altered Myocardial Microvascular 3D Architecture in Experimental Hypercholesterolemia

Martin Rodriguez-Porcel, Amir Lerman, Erik L. Ritman, Stephanie H. Wilson, Patricia J. M. Best and Lilach O. Lerman

_Circulation_. 2000;102:2028-2030
doi: 10.1161/01.CIR.102.17.2028

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/102/17/2028