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.

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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.
The micro-CT scanner has been described previously.\textsuperscript{5,6} 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,\textsuperscript{3} consisted of cubic voxels of 21 μm on a side and were displayed at 41 μm. 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 3-dimensionally oriented to obtain anatomically comparable samples for study. Subsequently, the myocardium was divided into 3 equal parts, and data were analyzed in 7 slices obtained at equal intervals from each third. In line with coronary artery distribution and regulation,\textsuperscript{8} 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 to 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 $P \leq 0.05$.

**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 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).

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**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 cm$^3$. *$P<0.05$ 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, particularly 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 responsible for coronary vascular resistance. This was associated with, and possibly provoked by, a small but significant decrease in the number of the upstream large microvessels. Several mechanisms may account for the decrease of large microvessels. HC is characterized by decreased bioavailability of nitric oxide, which may increase basal vascular tone and the wall thickness/lumen diameter ratio and could primarily affect microvessels and make their lumen appear smaller. The vascular frequency distribution remained unaltered, suggesting a relatively uniform effect on large microvessels. Alternatively, HC may involve enhanced release of substances stimulating extravascular matrix deposition, thereby speculatively expanding extravascular space and decreasing vascular density. However, the cardiac size and weight in this model are indistinguishable from control and thus unlikely to involve substantial expansion of extravascular space. Last, the luminal diameter of the larger microvessels may have declined because of augmented residual basal vascular tone. However, the changes in microvascular architecture in HC are more likely structural in type, because cold preservation during sample preparation results in almost complete muscle relaxation.

The HC-induced alterations in microvascular density may play a role in regulation and/or spatial distribution of myocardial perfusion. Newly generated microvessels may exhibit structural and functional abnormalities compared with native vessels. Hence, regulation of nutrient delivery to the myocardium in HC may be altered.

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
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References
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