Impaired myocardial perfusion reserve in experimental hypercholesterolemia is independent of myocardial neovascularization

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Mannheim D, Versari D, Daghini E, Gössl M, Galili O, Chade A, Rajkumar VS, Ritman EL, Lerman LO, Lerman A. Impaired myocardial perfusion reserve in experimental hypercholesterolemia is independent of myocardial neovascularization. Am J Physiol Heart Circ Physiol 292: H2449–H2458, 2007. First published January 5, 2007; doi:10.1152/ajpheart.01215.2006.—Our objective was to investigate the functional role of hypercholesterolemia-associated myocardial neovascularization in early atherosclerosis using the antiangiogenic thalidomide. Experimental atherosclerosis is characterized by myocardial neovascularization, associated with a decrease in myocardial perfusion response to challenge, coronary endothelial dysfunction, and high oxidative stress. However, the functional significance of these neovessels is not known. Three groups of pigs (n = 6 each) were studied after 12 wk of normal or hypercholesterolemic diet without (HC) or with thalidomide (HC + Thal). Myocardial perfusion and permeability were assessed at baseline and in response to cardiac challenge, using electron beam computed tomography, and coronary endothelial function was assessed using organ chambers. Myocardial samples were scanned ex vivo with a three-dimensional microscopic computed tomography scanner, and the spatial density of the myocardial microvessels was quantified. Growth factors and oxidative stress were measured in the myocardial tissue. As a result of these procedures, myocardial perfusion response to adenosine and dobutamine was blunted in both HC and HC + Thal pigs compared with normal pigs (P < 0.05, HC and HC + Thal vs. normal) as was the coronary endothelial function. Myocardial permeability response to adenosine was increased in both HC and HC + Thal pigs compared with normal pigs (P < 0.05, HC + Thal vs. normal, and HC + Thal vs. HC). The microvascular density was increased in HC pigs compared with normal pigs but normalized in HC + Thal pigs (P < 0.001 HC vs. normal and HC + Thal). HC + Thal pigs showed decreased expression of Flk-1 and basic FGF but increased expression of VEGF compared with normal and HC pigs. Oxidative stress was increased in both HC and HC + Thal pigs compared with normal pigs. In conclusion, chronic administration of thalidomide attenuates myocardial neovascularization in experimental HC pigs without affecting myocardial perfusion response to stimulation. This suggests that the myocardial neovascularization may not contribute to the attenuated myocardial perfusion response in hypercholesterolemia.

microcirculation; angiogenesis; thalidomide

IN ANIMAL MODELS of early atherosclerosis, cardiovascular risk factors, such as hypercholesterolemia and hypertension, are associated with myocardial neovascularization, related to the upregulation and increased activity of proangiogenic growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (30, 32, 44). We have previously demonstrated that in experimental hypercholesterolemia and hypertension, myocardial neovascularization is associated with impaired myocardial perfusion and permeability responses, as well as coronary endothelial dysfunction and an increase in oxidative stress (29, 32, 38). However, the role of the myocardial neovascularization in myocardial perfusion independently of oxidative stress is not well defined. Moreover, administration of high-dose vitamin C and E in experimental hypercholesterolemia prevents myocardial neovascularization while preserving myocardial perfusion and endothelial function and decreasing oxidative stress (29, 31, 44).

The present study was designed to investigate the functional role of the newly formed myocardial microvessels in early atherosclerosis, hypothesizing that these vessels are dysfunctional and contribute little to myocardial perfusion. For this purpose, hypercholesterolemic pigs were treated for 12 wk with thalidomide, a potent inhibitor of angiogenesis induced by VEGF (18) and b-FGF (7), known to decrease the number of microvessels and to increase the diameter of the remaining vessels in an animal tumor model (1). Moreover, the known antiangiogenic effects of thalidomide are not accompanied by a decrease in oxidative stress, as seen with statins and antioxidant vitamins (12, 26, 37), allowing a separation between the angiogenesis and oxidative stress. Subsequently, the association between attenuated microvascular neovascularization and myocardial perfusion in vivo and endothelial function in vitro was studied.

MATERIALS AND METHODS

All procedures were approved by the Institutional Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996).

Three groups of female domestic pigs (55–65 kg, 6 mo of age; n = 6 each) were studied: 1) normal, pigs fed a normal diet; 2) hypercholesterolemic (HC), pigs fed a high-cholesterol diet (2% cholesterol and 15% lard by weight; TD93296, Herlan Teklad); and 3) HC + Thal, pigs fed a HC diet with oral thalidomide (4 mg·kg⁻¹·day⁻¹, Celgene; NJ, gift from Celgene). This dose has been shown to cause a decrease in bone marrow microvascular density and is the recommended dose for treatment of multiple myeloma (8). After 12 wk, blood was collected for spectrophotometric measurement of plasma lipid profile (Roche, Nutley, NJ) and prostaglandin F₂α, isoprostane that was determined with an enzyme immunoassay kit (Cat. No. 516351, Cayman Chemical, Ann Arbor, MI) as previously described.
levels were selected. After 15 min of stabilization with intravenous gantry. With the use of localization scans, two contiguous mid-LV EBCT (Imatron c-150, Imatron, South San Francisco, CA) scanning ment of mean arterial pressure (MAP) and in the right atrium for catheters were placed fluoroscopically in the aorta for measurement of myocardial perfusion, permeability, and left ventricular (LV) ejection fraction (LVEF) as previously described (2, 3, 28, 29, 32).

In addition, segments of left circumflex artery were dissected for ex vivo analysis of the myocardial microvessels, as previously described (30, 44). Sleepaway, Fort Dodge Laboratories, Fort Dodge, IA), and the heart was then euthanized with intravenous pentobarbital sodium (20 ml of saline), intubated, and mechanically ventilated. A bolus of iopamidol (0.3 ml/kg, Isovue-370, Squibb Diagnostics, Princeton, NJ) was injected into the right atrium, and forty consecutive end-diastolic scans were obtained at intervals of one to three heart beats. Scans were obtained at baseline, and the study was repeated after 15 min of rest during a 10-min cardiac challenge with intravenous adenosine, titrated to the maximal dose possible while maintaining hemodynamic stability (~400 μg·kg⁻¹·min⁻¹). After 15 min of additional stabilization, intravenous dobutamine (5 to 25 μg·kg⁻¹·min⁻¹, until heart rate of 150 beats/min) was given, and the EBCT study was repeated after hemodynamic stabilization. LVEF was determined 15 min after administration of dobutamine with eight end-diastolic tomographic scans from LV apex to base during intravenous injection of contrast.

Regions of interest were traced in the LV cavity (for input function) and in the anterior cardiac wall (22). The average tissue opacity during the transit of contrast medium was then measured in each region of interest and plotted against time, yielding time-density curves. Each time-density curve was depicted as its two component curves, representing the transit of contrast medium through the intravascular and extravascular compartments of the myocardium, using extended gamma-variate curve-fitting algorithm (22). This approach enables independent calculation of perfusion and permeability (22, 29).

Intramyocardial vascular blood volume (blood volume, ml blood/ml tissue) and mean transit time were calculated by using saline, a bolus of iopamidol (0.3 ml/kg, Isovue-370, Squibb Diagnostics, Princeton, NJ) was injected into the right atrium, and forty consecutive end-diastolic scans were obtained at intervals of one to three heart beats. Scans were obtained at baseline, and the study was repeated after 15 min of rest during a 10-min cardiac challenge with intravenous adenosine, titrated to the maximal dose possible while maintaining hemodynamic stability (~400 μg·kg⁻¹·min⁻¹). After 15 min of additional stabilization, intravenous dobutamine (5 to 25 μg·kg⁻¹·min⁻¹, until heart rate of 150 beats/min) was given, and the EBCT study was repeated after hemodynamic stabilization. LVEF was determined 15 min after administration of dobutamine with eight end-diastolic tomographic scans from LV apex to base during intravenous injection of contrast.

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Intramyocardial vascular blood volume (blood volume, ml blood/ml tissue) and mean transit time were calculated by using systemic hemodynamics at baseline and in response to adenosine and dobutamine,jection fraction, plasma lipid profile, and isoprostanones in normal and hypercholesterolemic (HC) pigs and hypercholesterolemic pigs treated with thalidomide (HC + Thal) at 12 wk. MAP, mean arterial pressure. *P < 0.05 vs. normal.

| Table 1. Hemodynamic, plasma lipid, and isoprostanones profile |
|---------------|----------------|----------------|
|               | Normal         | HC             | HC + Thal      |
| n             | 6              | 6              | 6              |
| **Systemic hemodynamics** |               |                |                |
| MAP, mmHg     | 110 (SD 10)    | 114 (SD 16)    | 102 (SD 23)    |
| Heart rate, beats/min | 73 (SD 9) | 68 (SD 13) | 68 (SD 13) |
| **Adenosine** |                |                |                |
| MAP, %change  | −11 (SD 8)     | −15 (SD 12)    | −14 (SD 12)    |
| Heart rate, %change | 20 (SD 17) | 13 (SD 15) | 12 (SD 19) |
| **Dobutamine** |                |                |                |
| MAP, %change  | −7 (SD 8)      | 5 (SD 21)      | 11 (SD 14)     |
| Heart rate, %change | 126 (SD 14) | 133 (SD 50) | 145 (SD 11) |
| Ejection fraction, % | 54 (SD 3) | 57 (SD 5) | 56 (SD 6) |
| **Lipid profile** |                |                |                |
| Total cholesterol, mmol/l | 2.1 (SD 0.3) | 10.2 (SD 4.2)* | 9.1 (SD 3.0)* |
| LDL, mmol/l    | 1.0 (SD 0.2)   | 7.4 (SD 3.9)*  | 6.5 (SD 2.4)*  |
| HDL, mmol/l    | 1.0 (SD 0.2)   | 2.6 (SD 0.5)*  | 2.5 (SD 0.8)*  |
| Iso prostanes, pg/ml | 219 (SD 98) | 496 (SD 233)* | 563 (SD 254)* |

Values are means ± SD; n, number of pigs. Microvascular function: EBCT. EBCT was used for in vivo assessment of myocardial perfusion, permeability, and left ventricular (LV) ejection fraction (LVEF) as previously described (2, 3, 28, 29, 32). Briefly, the animals were anesthetized with intramuscular ketamine and xylazine (20 mg/kg and 2 mg/kg, respectively), with a maintenance dose of intravenous ketamine and xylazine (0.2 mg/kg and 0.03 mg/kg, respectively, in saline), intubated, and mechanically ventilated. Catheters were placed fluoroscopically in the aorta for measurement of mean arterial pressure (MAP) and in the right atrium for contrast medium injections. Animals were then placed supine in the EBCT (Imatron c-150, Imatron, South San Francisco, CA) scanning gantry. With the use of localization scans, two contiguous mid-LV levels were selected. After 15 min of stabilization with intravenous saline, a bolus of iopamidol (0.3 ml/kg, Isovue-370, Squibb Diagnostics, Princeton, NJ) was injected into the right atrium, and forty consecutive end-diastolic scans were obtained at intervals of one to three heart beats. Scans were obtained at baseline, and the study was repeated after 15 min of rest during a 10-min cardiac challenge with intravenous adenosine, titrated to the maximal dose possible while maintaining hemodynamic stability (~400 μg·kg⁻¹·min⁻¹). After 15 min of additional stabilization, intravenous dobutamine (5 to 25 μg·kg⁻¹·min⁻¹, until heart rate of 150 beats/min) was given, and the EBCT study was repeated after hemodynamic stabilization. LVEF was determined 15 min after administration of dobutamine with eight end-diastolic tomographic scans from LV apex to base during intravenous injection of contrast.

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| Table 2. EBCT-derived myocardial perfusion and permeability |
|---------------|----------------|----------------|
|               | Normal         | HC             | HC + Thal      |
| n             | 6              | 6              | 6              |
| **Perfusion** |                |                |                |
| Baseline, ml·min⁻¹·g⁻¹ | 0.82 (SD 0.21) | 0.95 (SD 0.20) | 1.29 (SD 0.22) |
| Adenosine, ml·min⁻¹·g⁻¹ | 1.2 (SD 0.19) | 0.95 (SD 0.19) | 1.17 (SD 0.26) |
| Dobutamine, ml·min⁻¹·g⁻¹ | 41.2 (SD 19.1) | 1.3 (SD 10.2)* | −9.1 (SD 15.3)* |
| **Dobutamine, %change** | 2.32 (SD 0.67) | 1.79 (SD 0.35) | 2.30 (SD 0.46) |
| Dobutamine, %change | 187.7 (SD 80.6) | 95.2 (SD 49.4)* | 90.1 (SD 34.9)* |
| **Permeability index** |                |                |                |
| Baseline, arbitrary units | 1.54 (SD 0.36) | 1.34 (SD 0.51) | 1.46 (SD 0.51) |
| Adenosine, arbitrary units | 1.73 (SD 0.52) | 2.29 (SD 0.58) | 1.77 (SD 0.40) |
| Dobutamine, arbitrary units | 11.2 (SD 12.1) | 80.0 (SD 33.2)* | 38.9 (SD 27.1)*† |
| Dobutamine, %change | 1.87 (SD 0.65) | 2.39 (SD 0.84) | 2.97 (SD 0.83) |
| Dobutamine, %change | 2.84 (SD 31.64) | 108.3 (SD 74.5)* | 133.9 (SD 74.7)* |

Values are means ± SD; n, number of pigs. Electron beam computed tomography (EBCT)-derived myocardial perfusion and permeability index response to adenosine and dobutamine in normal, HC, and HC + Thal pigs. *P < 0.05 vs. normal; †P < 0.05 vs. HC.

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Thalidomide treatment does not improve the blunted perfusion response to adenosine (top) and dobutamine (bottom). Responses are represented as percent change compared with baseline in normal pigs, hypercholesterolemic (HC) pigs, and HC pigs treated with thalidomide (HC + Thal). Thalidomide treatment does not improve the blunted perfusion response to challenge observed in HC pigs. *P < 0.05 vs. normal.

previously validated algorithms (22). Myocardial perfusion (in ml·min⁻¹·g⁻¹) was calculated as follows: 60 × (blood volume/mean transit time)/(1.05 × (1 – blood volume)), where 1.05 g/ml is the specific density of the myocardium. Coronary microvascular permeability index (in arbitrary units) was calculated as follows: [60 × 1.05 × (maximal slope of ascending part of extravascular curve × mean transit time)/area under left ventricular curve/blood volume] (29).

With the use of Analyze (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN), LV end-diastolic and end-systolic frames were identified from each cine-mode movie spanning the cardiac cycle at each level. LV end-diastolic and end-systolic areas were measured from apex to base and subsequently added by using a modified Simpson’s formula to determine the end-diastolic and end-systolic volumes. LVEF was calculated as (LV end-diastolic volume – LV end-systolic volume)/LV end-diastolic volume.

Microvascular architecture: microscopic computed tomography. Hearts were prepared as previously described (30, 44). Briefly, the proximal left anterior descending artery was cannulated in situ and perfused with a solution of 0.9% normal saline and heparin to flush out the remaining blood. An intravascular radiopaque microfil silicone rubber (MV-122, Flow Tech, Carver, MA) was injected at a physiological perfusion pressure (100 mmHg) until it flowed freely from the myocardial veins. A transmural portion of the LV (~2 × 1 × 1 cm³) was sectioned and scanned (30, 44).

The microscopic computed tomography scanner has been previously described (16, 30). Myocardial samples were scanned using 0.5° angular increments, providing 721 views around 360°. Images were recorded, digitized, and transferred to a controlling computer. The three-dimensional volume images consisted of cubic voxels, 20 μm on a side, and were displayed at this resolution. The radiopacity of each voxel was represented by a 16-bit grayscale value.

Image analysis was performed using Analyze software, as previously described (30, 44). The myocardium was three-dimensionally oriented to obtain anatomically comparable samples for study. Subsequently, the myocardium was divided into two equal parts, classified as subendocardium and subepicardium.

Microvessels (diameters < 500μm) were counted in each tomographic section using the “object counter” function and classified as either small (diameters between 40 and 100 μm) or large (diameters between 101 and 500 μm). Diameters of microvessels larger than 100 μm were measured manually (44). Forty micrometers (2 voxel) was considered the minimal detection limit, and microvessels smaller than 40 μm (<2 voxel) were excluded to avoid error due to noise. The area of myocardium in each slice was traced to calculate the vascular density per slice.

With the use of a “connectivity” function, three intramyocardial arterioles and their branches were isolated in each pig. The vessel elongation factor, representing vessel tortuosity (typical for neovascularization), was calculated using “tree analysis” software. Th elongation factor was calculated as follows: the total three-dimensional distance/the shortest distance between two end points of the main branches.

Endothelial function. Coronary endothelial function was assessed as previously described (13, 36). Briefly, segments of the left circumflex artery 2–3 cm long were dissected, sectioned into 0.5-cm-long rings, and placed in a modified Krebs solution. The rings were precontracted with 10⁻⁷ mol/l endothelin-1 (Phonix) (0% relaxation) and then relaxed with cumulative concentrations of endothelial-de-
ependent bradykinin \((10^{-11}-10^{-6}\text{ mol/l, Sigma Chemical})\) or nonreceptor-mediated endothelium-dependent vasodilator calcium ionophore A-23187 \((10^{-11}-10^{-6}\text{ mol/l, Sigma})\) or endothelial-independent sodium nitroprusside \((10^{-11}-10^{-6}\text{ mol/l})\). At the end, \(10^{-3.5}\text{ mol/l papaverine (Sigma)}\) were added to determine the maximal vasodilatory capacity (100% relaxation) (13).

Coronary artery relaxation was expressed as percent relaxation (related to 100% relaxation with papaverine). At least five rings were used in each group, and no more then two rings from each animal per group.

**Detection of superoxide anion.** In situ production of superoxide anion was measured in 30-μm frozen myocardium sections using the oxidative fluorescent dye dihydroethidium, as previously described (5, 43). Dihydroethidium appears with a blue fluorescence in the cytosol, but when oxidized by superoxide to ethidium bromide, it intercalates in the cell DNA of the cell, staining the nucleus a fluorescent red (excitation at 488 nm and emission at 610 nm). Serial sections were equilibrated under identical conditions for 30 min at 37°C in Krebs-HEPES buffer. Fresh dihydroethidium (2 μmol/l) was applied, incubated for 30 min in a dark humidified chamber at 37°C, and evaluated under fluorescence microscopy. MetaMorph imaging program (Meta Imaging series 4.6) was used to calculate the percentage of red stained cells per field. Two slides were stained per pig. Five fields were sampled in every slide.

**Immunohistochemistry for nitrotyrosine.** After being deparaffinized and hydrated, slides were incubated overnight at 4°C with primary antibody (mouse monoclonal; Cayman; 1:50) and then 1 h with secondary antibody. Diaminobenzidine was used as chromogen. Slides were viewed by microscope (Olympus, Leeds Precision Instruments) and analyzed with MetaMorph imaging software. The percentage of staining per field was semiautomatically quantified. Two slides were stained per pig. Three fields were sampled in every slide.

**Fig. 3.** Representative three-dimensional tomographic images (20 μm voxel size) of myocardium from normal, HC, and HC + Thal pigs. Top: transmural myocardium. Bottom: tomographically isolated transmural arterioles, showing that thalidomide treatment decreases the microvascular sprouting and tortuosity typical to HC.

**Table 3. Microvascular architecture**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>HC</th>
<th>HC + Thal</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(n)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><strong>Subepicardial</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small (≤100 μm)</td>
<td>105 (SD 86)</td>
<td>294 (SD 67)*</td>
<td>89 (SD 20)</td>
</tr>
<tr>
<td>Large (101-500 μm)</td>
<td>28 (SD 22)</td>
<td>32 (SD 15)</td>
<td>24 (SD 4)</td>
</tr>
<tr>
<td><strong>Subendocardial</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small (≤100 μm)</td>
<td>162 (SD 140)</td>
<td>332 (SD 96)*</td>
<td>125 (SD 37)</td>
</tr>
<tr>
<td>Large (101-500 μm)</td>
<td>38 (SD 29)</td>
<td>30 (SD 11)</td>
<td>29 (SD 5)</td>
</tr>
<tr>
<td>Mean diameter of large vessels, μm</td>
<td>159 (SD 18)</td>
<td>152 (SD 10)</td>
<td>179 (SD 11)*</td>
</tr>
<tr>
<td>Elongation factor</td>
<td>1.3 (SD 0.1)</td>
<td>1.6 (SD 0.1)*</td>
<td>1.2 (SD 0.1)</td>
</tr>
</tbody>
</table>

Values are means ± SD; \(n\), number of pigs. Spatial density (vessels/cm²) and mean vascular diameter of myocardial microvessels in normal, hypercholesterolemic (HC), and hypercholesterolemic treated with thalidomide (HC + Thal). \(*P < 0.05\) vs. normal and HC + Thal; \(\dagger P < 0.05\) vs. normal and HC.
Western blot analysis. Freshly frozen tissue was homogenized, and protein concentrations were calculated by Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of myocardial homogenate protein (100 μg) were dissolved in SDS-polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes (Bio-Rad). Membranes were incubated overnight at 4°C with either anti-VEGF (mouse monoclonal 1:500, Novus Biologicals, Littleton, CO), anti-b-FGF (rabbit polyclonal 1:100, Santa Cruz Biotechnology, Santa Cruz, CA), or anti-Flk-1 (rabbit polyclonal 1:100, Santa Cruz Biotechnology), as previously described (32, 44). The appropriate horseradish peroxidase-linked secondary antibody was then used. The proteins were detected by electrochemiluminescence. β-Actin was used as the loading control.

Statistical analysis. Data are expressed as means (SD). Comparisons between the groups were performed using ANOVA. Statistical significance was accepted for \( P < 0.05 \).

RESULTS

Systemic hemodynamics. Basal MAP, heart rate, and LVEF were similar in all groups (Table 1).

Plasma lipid profile. After 12 wk of high-cholesterol diet, there was a significant increase in plasma cholesterol (total, low-density lipoprotein, and high-density lipoprotein) with a similar lipid profile in the cholesterol-fed pigs (HC and HC + Thal groups) compared with normal pigs (Table 1).
MYOCARDIAL NEOVESSELS IN HYPERCHOLESTEROLEMIA

Myocardial vascular function. During the EBCT study, the change in MAP and heart rate in response to adenosine and dobutamine was similar among the groups (Table 1). Baseline perfusion of the anterior myocardial wall was higher in the HC + Thal group compared with normal and HC groups (Table 2). The perfusion response to adenosine was blunted in HC and HC + Thal pigs compared with normal pigs with no significant difference between HC and HC + Thal pigs (Table 2 and Fig. 1, top). The change in perfusion response to dobutamine stimulus was attenuated in both HC groups compared with the normal group, with no significant difference between HC and HC + Thal groups (Table 2 and Fig. 1, bottom).

Baseline permeability index was similar in all the study groups (Table 2). The change in permeability response to adenosine was significantly greater in HC and HC + Thal pigs compared with normal pigs and in HC pigs compared HC + Thal pigs (Table 2 and Fig. 2, top).

The change in permeability response to dobutamine was also significantly higher in HC and HC + Thal pigs compared with normal pigs, with no significant difference between the HC groups (Table 2 and Fig. 2, bottom).

Microvessel architecture. The transmural spatial density of microvessels (<500 μm) was significantly higher in HC pigs compared with normal pigs [344.3 (SD 64.7) vs. 129.3 vessels/cm² (SD 107.3) vessels/cm², P < 0.001; Fig. 3] but was preserved in the HC + Thal pigs and not different than normal pigs [129.8 vessels/cm² (SD 23.5), P = not significant (NS) vs. normal and P < 0.0001 vs. HC; Fig. 3]. This preservation of microvessel density was evident in small microvessels (<100 μm) both in the subepicardium and subendocardium, with no significant change in the density of the larger microvessels (101–500 μm) (Table 3). Elongation factor, a parameter of tortuosity, was also higher in HC pigs compared with normal pigs but normalized in HC + Thal pigs (Table 3). The mean diameter of the large vessels (101–500 μm) was significantly higher in HC + Thal pigs compared with normal and HC pigs (Table 3).

Myocardial oxidative stress. Myocardial oxidative stress was evaluated by tissue superoxide production and nitrotyrosine immunostaining. Superoxide production was similar in both HC and HC + Thal pigs [3.8% (SD 1.4) and 3.4% stained cells/field (SD 1.4), respectively; P = NS] and higher compared with normal pigs [1.1% (SD 0.6) stained cells/field, P < 0.05 for both] (Fig. 4, top). Nitrotyrosine immunostaining was also similar in HC and HC + Thal pigs [23.5% (SD 10.5) and 21.2% stained cells/field (SD 10), respectively; P = NS] and higher compared with normal pigs [4.9% stained cells/field (SD 2.2), P < 0.05 for both] (Fig. 4, bottom).

Systemic oxidative stress, evaluated by plasma isoprostanes, was also higher in both HC and HC + Thal pigs compared with normal pigs, with no difference between HC and HC + Thal pigs (Table 1).

Vascular endothelial function. Coronary arteries from HC pigs showed a significantly attenuated vasorelaxation response to the endothelial-dependent vasodilator bradykinin compared with those from normal pigs [mean maximal relaxation: HC, 61.0% (SD 17.3); and normal, 90.5% (SD 8.4), P < 0.001]. There was a similar impairment in maximal vasodilatation in response to bradykinin in the HC + Thal group with no significant difference versus the HC group [mean maximal relaxation: HC + Thal, 47.55% (SD 17.0), P < 0.001 vs. normal] (Fig. 5, top).

Calcium ionophore caused a similar maximal vasorelaxation in all three groups [normal, 95.3% (SD 9.3); HC, 85.8% (SD 13.1); and HC + Thal, 88% (SD 6.6), P = NS].

The maximal relaxation response of the epicardial arteries to endothelial-independent vasodilator sodium nitroprusside was similar in all study groups [normal, 79.6% (SD 13); HC, 86.4% (SD 7.6); and HC + Thal, 84.5% (SD 11.2), P = NS] (Fig. 5, bottom).

Myocardial expression of growth factors. Myocardial expression of VEGF receptor 2 (Flk-1), VEGF, and b-FGF was significantly decreased in HC pigs compared with normal HC pigs (Fig. 6, top). VEGF was significantly higher in HC compared with normal pigs and further increased in HC + Thal compared with HC and normal pigs (Fig. 6, bottom). On the other hand, b-FGF expression was significantly higher in HC pigs [0.44 (SD 0.22)] compared with both normal and HC + Thal pigs [normal, 0.21 (SD 0.04); and HC + Thal, 0.22 (SD 0.07); P < 0.05] without a significant difference between normal and HC + Thal pigs (P = NS).

DISCUSSION

The present study demonstrates that attenuating neovascularization associated with hypercholesterolemia does not improve myocardial perfusion reserve when endothelial dysfunc-
tion and oxidative stress are not improved as well. The current study suggests that the myocardial neovascularization associated with hypercholesterolemia does not contribute to myocardial perfusion reserve and that the blunted perfusion response associated with hypercholesterolemia might be related to endothelial dysfunction and oxidative stress. This study also demonstrates that the antiangiogenic effect of thalidomide was at least partly mediated by a decrease in Flk-1 expression.

Experimental hypercholesterolemia is associated with myocardial neovascularization characterized by small, tortuous microvessels (29), as well as micro- and macrovessel (epicardial vessels) endothelial dysfunction (38), both of which may contribute to a blunted myocardial perfusion response to challenge. This angiogenesis is likely mediated by the increase in proangiogenic growth factors such as VEGF and b-FGF observed in the myocardium in experimental hypercholesterolemia and hypertension (32, 44). The local increase in these growth factors may be triggered by the increase in oxidative stress and hypoxia-induced factor-1α (HIF-1α) seen in these animals (29, 44), as well as directly by oxidized low-density lipoprotein-induced VEGF mRNA stability (33).

Lowering oxidative stress with vitamins or statins in this model of HC was accompanied by a decrease in HIF-1 and growth factors as well as a normalization of the myocardial microvascular architecture and an improvement in endothelial function (31, 41, 44). The concomitantly attenuated angiogenesis and improved endothelial function of the remaining vessels (both micro and macro) was associated with an overall improvement in the myocardial perfusion response to challenge (3, 28).

To examine the contribution of the neovasculature to the myocardial perfusion, we used thalidomide that inhibits both VEGF- and b-FGF-induced angiogenesis (7, 18), which has been shown to be important contributors to hypercholesterolemia-associated neovascularization, without decreasing oxidative stress (12, 26, 37).

In the present study, a long-term administration of thalidomide in experimental hypercholesterolemia was associated with attenuated myocardial neovascularization despite an apparent paradoxical increase expression of VEGF. A similar increase in VEGF was seen in previous studies examining the effect of thalidomide on bone marrow microvascular density in multiple myeloma patients (8, 15) or using specific anti-VEGF agents in a model of neuroblastoma (17).

Downregulation of Flk-1 expression in the myocardium observed in this study, and previously demonstrated (42), may have prevented the VEGF signal transduction and may be one of the mechanisms for the attenuated neovascularization observed in the thalidomide-treated animals. The concomitant increase in VEGF might be attributed to an upregulation of VEGF by positive feedback, as also observed in patients with chronic coronary heart disease and diabetes (35).
dial and systemic oxidative stress that did not diminish by the addition of thalidomide could be the drive for the increase in VEGF expression in both hypercholesterolemic groups and further stresses the importance of the VEGF proangiogenic pathway in the angiogenesis associated with hypercholesterolemia. Another mechanism for the attenuated neovascularization could be the decrease in b-FGF; another potent regulator of angiogenesis (7, 39).

As previously demonstrated (3, 29), in this study hypercholesterolemia impaired myocardial perfusion and permeability with associated endothelial dysfunction and an increase in oxidative stress (29, 38, 41, 44). A long-term administration of thalidomide with a HC diet was associated with a decrease in myocardial microvessels and their tortuosity. Unlike previous studies with antioxidant vitamins and statins, this angiogenic effect did not involve a decrease in oxidative stress or an improvement in endothelial function, allowing us to examine separately the effect of these microvessels on myocardial perfusion. This isolated normalization of myocardial architecture was associated with a blunted myocardial perfusion response, suggesting that the small, tortuous, and dysfunctional neovessels associated with hypercholesterolemia did not substantially contribute to or interfere with myocardial perfusion and constituted ineffective compensation for the vascular functional abnormalities. Indeed, we have recently shown that myocardial neovascularization in early hypertension also leads to the formation of similarly dysfunctional vessels (32).

Experimental hypercholesterolemia is also associated with microvascular hyperpermeability that is corrected by decreasing oxidative stress using statins or vitamins (3, 28, 29). In the current study, preventing the formation of the new microvessels without improving the endothelial function of the remaining vessels only partially corrected the increased permeability when challenged with adenosine, showing that the increased permeability seen in HC pigs is both functional (possibly due to oxidative stress) and structural (possibly due to increased porosity of the internal elastic lamina) (19). This correction was not evident during dobutamine challenge, possibly due to the more pronounced hemodynamic response elicited by dobutamine.

Another interesting finding of the present study is the higher baseline perfusion observed in the HC + Thal group, which might be in part responsible for the blunted response to adenosine and dobutamine. However, this level of perfusion was well below the maximal response to stimulation shown in previous studies (4.5 ml·min⁻¹·g⁻¹) (24). The mechanism of this increase may be related to the increase in mean vascular diameter of the large vessels seen in the HC + Thal group, which may imply an increase in the number of larger vessels or a vasodilatation of the remaining vessels. A similar increase in vessel diameter was observed in animal tumor models using thalidomide (1), or a specific VEGF receptor tyrosine kinase inhibitor, that caused a reduction in Flk-1 and microvessel with a concomitant increase in the number of large-diameter vessels and a decrease in blood flow velocity in the artery feeding the tumor (9). Another mechanism for the increase in baseline perfusion might be the high-VEGF expression, which has been demonstrated to have a vasodilatory effect (20, 23). In particular, the VEGF-induced vasodilatation was predominantly in the small myocardial arterioles (60–100 μm) with little effect on coronary arteries and was only partially inhibited by an inhibitor of nitric oxide production (N⁴-nitro-L-arginine). This vasodilatory effect could not be ascribed to the VEGF receptors since there is no difference in the distribution of VEGF receptors between the micro- and macromyocardial vessels (20). Since vessel diameter is measured ex vivo with vessels in almost complete relaxation (25), although the flow and perfusion are measure in vivo, the changes in vessel diameter are most likely due to a change in actual vessel size, whereas the increase in baseline perfusion could be a result of both an increase in vessel size or vasodilation.

Limitations. The magnitude of the perfusion response to intravenous dobutamine in normal animals was similar to that observed in healthy humans and pigs (6, 21). On the contrary, the increase in perfusion in response to intravenous adenosine in normal animals was less than that observed in previous studies in humans using intracoronary Doppler flow measurements (40). Using lower doses of adenosine has been shown to elicit mainly arteriole (<100 μm) vasodilatation, enabling us to examine the microvasculature response (27). Although the use of higher doses may elicit a more pronounced response, it also causes hemodynamic instability, hypoxia, and vasodilatation of larger vessels, making the measurements less targeted to the microcirculation.

Thalidomide is not a specific antiangiogenic drug; however, the myocardial structural modification seen in this study are in concordance with results of previous studies using specific VEGF receptor inhibitors (9). Thalidomide is also an immunomodulator, inhibiting secretion of tumor necrosis factor-α on the one hand (34) and costimulating T cells on the other hand (14). The significance of this anti-tumor necrosis factor-α effect has been recently challenged in a trial of thalidomide in chronic heart failure, where an improvement in cardiac output was noted despite an increase of tumor necrosis factor-α (10).

In conclusion, myocardial neovascularization in response to hypercholesterolemia is an ineffective form of compensation and does not contribute significantly to myocardial perfusion response to stimulation when oxidative stress and endothelial function are not improved. This study underscores the importance of addressing not only the number of vessels but also their microenvironment during design of proangiogenic strategies, especially now that inducing myocardial angiogenesis is emerging as a tempting therapy for patients with chronic myocardial ischemia (11).

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REFERENCES


