The three members of the family of endothelins are produced in a variety of tissues and are involved in the regulation of vasomotoric tone and cell proliferation (1). Notably, endothelin-1 (ET-1), the 21-amino acid peptide produced by endothelial cells, has been implicated in endothelial dysfunction in atherosclerosis and in neovascularization in malignancies (2,3). Within the vasculature, these effects of ET-1 are mediated by endothelin-A (ET-A) receptors on vascular smooth muscle cells and endothelin-B (ET-B) receptors on both vascular smooth muscle cells and endothelial cells (1,2).

Various factors such as the renin-angiotensin system (RAS) modulate the activity of the endogenous endothelin system, whereby angiotensin II (Ang II) stimulates the expression of both ET-1 and ET-A receptors (4–7). Of note, ET-A receptor antagonism inhibits this activation pathway of the endogenous endothelin system and reduces ET-1 vascular tissue levels (8,9). In cardiovascular disease and malignancies, endothelin receptor antagonism preserves biological integrity and reduces tumor neovascularization, respectively (3,10,11).

Experimental hypercholesterolemia is associated with enhanced vasoconstriction of the coronary circulation in association with increased vascular tissue expression of ET-1 (10–12). Moreover, using a novel imaging technique, we demonstrated that experimental hypercholesterolemia is also associated with an increase in the spatial density of coronary vasa vasorum (13,14). Notably, this neovascularization process occurs very early in the atherosclerotic process and before epicardial endothelial dysfunction (15).

Given the mitogenic effect of ET-1 upon endothelial cells (16), the stimulatory effect of ET-1 upon the release of vascular endothelial growth factor (VEGF) from vascular smooth muscle cells (17) and the correlation of ET-1 expression with microvessel density in tumor neovascularization (18), the endogenous endothelin system might potentially be involved in vasa vasorum neovascularization in early atherosclerosis. Thus, this study was designed to test the hypothesis that chronic endothelin receptor antagonism...
will preserve coronary vasa vasorum neovascularization in experimental hypercholesterolemia.

METHODS

Animals. The study was reviewed and approved by the Mayo Foundation Institutional Animal Care and Use Committee. For a study period of 12 weeks, female domestic pigs (weight: 25 kg to 35 kg) were placed on a normal chow diet (N; n = 7), on a hypercholesterolemic diet containing 2% cholesterol and 15% lard (TD 93296, Harlan Teklad, Madison, Wisconsin) (HC; n = 6) or on a hypercholesterolemic diet supplemented with selective ET-A receptor antagonist (HC + ET-A; n = 6) by weight-adapted, oral application of ABT-627 (4 mg/kg per day, Abbott Laboratories, Abbott Park, Illinois) as described in detail before (10,11). ABT-627 is the active R,R,S isomer of the racemic compound A-127722, acting as a competitive inhibitor of ET-A and ET-B receptor, respectively (19). At the end of each study period, blood samples were taken for analysis of plasma lipid levels, followed by euthanization of the animals.

Microcomputed tomography imaging. The left anterior descending coronary artery was prepared and scanned by microcomputed tomography as described by our group in previous studies (13–15). The imaging system yielded a matrix of 42 μm cubic voxels with a 16 bits gray scale and an average number of 500 slices per coronary artery segment for analysis (13–15). The resulting three-dimensional digitalized image was analyzed by using the Analyze software package (Biomedical Imaging Resource, Mayo Foundation, Rochester, Minnesota) (15).

All specimens were traced over their entire length, and cross-sections were analyzed at 1-mm intervals. Areas of potential source of error, such as branching points, were excluded from the analysis. Thus, six to 12 topographic cross-sections were analyzed for each specimen, on average. As reported before, vasa vasorum area was determined in each cross-section and designated vessel wall area (14,15,20,21). Vasa vasorum were manually traced and measured in this area, yielding the following parameters for each cross-section: vessel wall area, vasa vasorum count and vasa vasorum density (i.e., vasa vasorum per mm² vessel wall area), mean diameter of first and second order vasa vasorum and ratio of the number of second to first order vasa vasorum. First order vasa vasorum originated from the main coronary lumen and ran longitudinally to the coronary artery. Second order vasa vasorum originated from first order vasa vasorum and ran circumferentially around the lumen (14). Data analysis was performed in standardized, nonblinded manner among all experimental groups. Mean values were obtained for each individual animal, and data presentation per group was based on these values. Overall, the mean coefficients of variation for vessel wall area, vasa vasorum count and vasa vasorum density were 0.13, 0.45 and 0.38, respectively, without any significant differences among the groups.

Immunohistochemistry. After deparaffinization in xylene and rehydration in 100%, 95% and 70% ethanol, porcine coronary artery slides were incubated with equimolar 3% H₂O₂/100% MeOH solution to block endogenous tissue peroxidase activity. Anti-VEGF primary antibody (dilution 1:100, 4°C overnight, Santa Cruz Biotechnology Inc., Santa Cruz, California) was detected with the EnVision kit (Dako Corporation, Carpinteria, California) in peroxidase-labeling technique and 3,3-diaminobenzidine tetrahydrochloride (DAB) as chromogen (Vector Laboratories Inc., Burlingame, California) to yield a brownish reaction product. Incubation with an unspecified isotype antibody served as a control for the specificity of immunoreactivity. All sections were counterstained with hematoxylin.

Double-label immunohistochemistry. Double-label immunostaining of coronary artery specimens was performed using the EnVision Doublestain Kit (Dako). In the first step, anti-VEGF antibody (Santa Cruz) was detected by a peroxidase-labeled secondary antibody with DAB as chromogen (Vector Laboratories). In the second step, anti-smooth muscle alpha-actin (dilution 1:1500; Dako) antibody was detected by an alkaline phosphatase-labeled secondary antibody with Vector Red as chromogen (Vector Laboratories). Endogenous alkaline phosphatase was blocked by incubation with levamisole (Dako). All sections underwent counterstaining with hematoxylin.

Western blot. After removal of the heart, coronary arteries were snap frozen in liquid nitrogen and stored at −80°C until further processing. All tissues were homogenized using a tissue homogenizer and a lysis buffer of the following composition: 50 nM Tris HCl, pH 8.0, 150 mM NaCl, 0.02% Sodium Azide, 0.1% SDS, PMSF 100 μg/ml, Aprotinin 1 μg/ml 1% NP-40, 0.5% sodium deoxycholate. The lysate was analyzed for protein content using a Bradford assay (Bio-Rad Laboratories, Hercules, California), and equal amounts of protein were resolved under reducing conditions on an 8% SDS-polyacrylamide gel. Immuno-
Table 1. Micro-CT Parameter

<table>
<thead>
<tr>
<th></th>
<th>N (n = 7)</th>
<th>HC (n = 6)</th>
<th>HC + ET-A (n = 6)</th>
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<tbody>
<tr>
<td>Vessel diameter (mm)</td>
<td>1.8 ± 0.5</td>
<td>1.9 ± 0.5</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>Lumen area (mm²)</td>
<td>2.6 ± 1.2</td>
<td>2.9 ± 1.3</td>
<td>3.0 ± 0.8</td>
</tr>
<tr>
<td>Vessel wall area (mm²)</td>
<td>1.8 ± 0.7</td>
<td>2.8 ± 1.3</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>Vasa vasorum count (n)</td>
<td>4.9 ± 3.6</td>
<td>11.7 ± 4.7*</td>
<td>6.7 ± 2.3</td>
</tr>
<tr>
<td>Vasa vasorum/vessel wall area (n/mm²)</td>
<td>2.5 ± 1.5</td>
<td>4.7 ± 1.8*</td>
<td>3.2 ± 0.7</td>
</tr>
<tr>
<td>Vasa vasorum/lumen area (n/mm²)</td>
<td>1.7 ± 1.1</td>
<td>4.5 ± 1.3</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>Ratio 2nd/1st order vasa vasorum</td>
<td>2.6 ± 2.0</td>
<td>3.7 ± 1.3</td>
<td>2.3 ± 1.9</td>
</tr>
<tr>
<td>Diameter 1st order vasa vasorum (µm)</td>
<td>96 ± 32</td>
<td>93 ± 12</td>
<td>81 ± 10</td>
</tr>
<tr>
<td>Diameter 2nd order vasa vasorum (µm)</td>
<td>62 ± 6</td>
<td>55 ± 6</td>
<td>58 ± 7</td>
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</table>

N, HC, HC + ETA = animals fed a normal diet or a high-cholesterol diet without or with endothelin-type A-receptor antagonism for 12 weeks, respectively. Parameter values are mean ± SD; †p < 0.05 vs. N; ‡p < 0.05 vs. N and HC + ET-A.

RESULTS

Animals. Compared with N animals, plasma lipid concentrations were higher in the HC and HC + ET-A animals (total cholesterol: 84 ± 3 mg/dl, 497 ± 164 mg/dl, 428 ± 124 mg/dl; LDL: 36 ± 3 mg/dl, 312 ± 153 mg/dl, 303 ± 113 mg/dl; HDL: 41 ± 5 mg/dl, 99 ± 38 mg/dl, 117 ± 13 mg/dl; p < 0.05 N vs. HC and HC + ET-A for total cholesterol, low-density lipoprotein and high-density lipoprotein). No significant difference in systemic hemodynamics was observed between the groups (11).

Microcomputed tomography imaging. Vasa vasorum count and vasa vasorum density were higher in the HC than in N pigs but similar in the HC + ET-A and N pigs (Table 1). In N pigs, the spatial distribution pattern of coronary vasa vasorum was clearly structured into first order vasa vasorum, which run longitudinally to the main lumen, and second order vasa vasorum, which run circumferentially around the main lumen. In HC animals, this characteristic structure was abolished, whereas, in HC + ET-A pigs, the spatial distribution pattern of coronary vasa vasorum was similar to N animals (Fig. 1).

Immunohistochemistry. Immunoreactivity for VEGF was present predominantly in the outer media and, to a lesser extent, in the adventitia. In the adventitia, VEGF immunoreactivity co-localized with endothelial cells of the vasa vasorum in all groups but was also found in the connective tissue in the adventitial area adjacent to the media in HC animals. In this study group, immunoreactivity for VEGF was most intense and co-localized preferentially with vascular smooth muscle cells in the outer media (Fig. 2).

Figure 1. Representative microcomputed tomography images of the proximal segments of the left anterior descending artery from animals fed a normal diet (N) or high cholesterol diet without (HC) or with endothelin-type A-receptor antagonism (HC + ET-A) for 12 weeks. The spatial distribution pattern of vasa vasorum in the N group is characterized by a clear structure into first order vasa vasorum, running longitudinally and second order vasa vasorum, running circumferentially. In the HC group, a plexus of newly formed vasa vasorum and in the HC + ET-A group a restitution to a normal status are apparent. Illustration voxel size 21 µm.
Western blotting. Quantitative analysis by Western blotting demonstrated that the amount of VEGF within the coronary arterial wall was higher in the HC group than in the N group and significantly lower in HC/ET-A pigs than in HC pigs (p < 0.05) (Fig. 3).

DISCUSSION

The principal finding of this study is that chronic antagonism of the endothelin type A receptor prevents the increase in the spatial density of vasa vasorum of epicardial arteries in experimental hypercholesterolemia. This effect of ET-A antagonism upon vasa vasorum structure was associated with similar preservation of VEGF expression in the coronary arterial wall and was observed without changes in plasma lipid values. Thus, this study supports a role for the endogenous endothelin system in the development of coronary vasa vasorum neovascularization in early coronary atherosclerosis.

Endothelin and tumor neovascularization. Increased tissue levels of ET-1 have been demonstrated in a variety of different tumors, and ET-1 has been identified as an autocrine growth factor and angiogenic factor in these tissues (3). Moreover, the expression of ET-1 in tumor...
tissue has been shown to correlate with malignancy and vascularity in colorectal cancer and brain tumors (3). Salani et al. (18) reported on the correlation between tumor microvessel count and ET-1 expression in ovarian carcinoma. Furthermore, they found a correlation between ET-1 and VEGF expression in these tissues, as assessed by the use of immunohistochemical methods, and suggested that both ET-1 and VEGF contribute to tumor neovascularization (18). Indeed, in vitro studies, they clearly demonstrated that ET-1 enhances VEGF-induced neovascularization (18,22). Of note, stimulation of VEGF production of ovarian carcinoma cells by ET-1 was completely blocked by an ET-A antagonist (18). Thus, a role for the endogenous endothelin system in tumor neovascularization has been increasingly recognized during recent years.

**Endothelin and coronary neovascularization.** Increase in tissue expression of ET-1 has also been described in the vascular wall in preatherosclerotic and atherosclerotic disease states, including experimental hypercholesterolemia (12). As with tumor vessels, it has been shown that vascular smooth muscle cells of macro- and microvessels of the coronary circulation express both ET-A and ET-B receptors, whereas endothelial cells exclusively express ET-B receptors (23,24). In addition to a direct mitogenic effect upon endothelial cells, mediated by the ET-B receptor, in vitro studies demonstrated that ET-1 can lead to endothelial cell proliferation, migration and invasion of the extracellular matrix by stimulation of the synthesis of VEGF in vascular smooth muscle cells, mediated by the ET-A receptor (17,25,26). In this study, coronary artery expression of VEGF increased during experimental hypercholesterolemia, predominantly in the smooth muscle cell layer of the media. Of note, this increase in VEGF expression by medial smooth muscle cells was prevented by ET-A receptor antagonism as was increase in spatial density of coronary vasa vasorum during experimental hypercholesterolemia. Thus, this study, for the first time, supports a pathophysiologic role for the endogenous endothelin system in coronary vasa vasorum neovascularization in experimental hypercholesterolemia.

**Endothelin, the RAS and oxidative stress.** The role of other vasoactive peptides cannot be ruled out. In this regard, an important role in the regulation of ET-1 expression has been attributed to the RAS (4,27). Indeed, experimental hypercholesterolemia is characterized by an increase in the expression of angiotensin type 1 receptor and, therefore, by an enhancement of the Ang II pathway on vascular tissue level (28). Given the stimulatory action of Ang II upon the

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**Figure 3.** Bar graph illustrating vascular endothelial growth factor expression in the coronary arterial wall from animals fed a normal diet (N, n = 4) or high cholesterol diet without (HC, n = 3) or with endothelin-type-A receptor antagonism (HC + ET-A, n = 3) for 12 weeks. Vascular endothelial growth factor protein content was higher in the HC group than in the N group (p = 0.06) and significantly lower in the HC + ET-A group than in the HC group. *p < 0.05. Values are mean ± standard error.
expression of ET-1, this might translate into an increase in vascular tissue levels of ET-1, which has, in fact, been demonstrated in prior in-vivo studies (8,9). Importantly, ET-A receptor antagonism abolishes this stimulatory action of Ang II and reduces vascular tissue levels of ET-1 (8,9). Furthermore, the local RAS might stimulate vascular tissue expression of ET-1 by increase in endogenous oxidative stress (29–31). Thus, by virtue of the fact that ET-1 is a potent stimulator of VEGF expression, ET-A receptor antagonism might prevent an increase in vascular tissue expression of VEGF and an increase in vasa vasorum spatial density by reducing tissue levels of ET-1 itself. In this regard, interference with the stimulatory action of the local RAS and even with autocrine mechanisms of stimulation, has to be considered as a potential mode of action (32).

Endothelin and vascular wall perfusion. In addition to the stimulation of VEGF production in vascular smooth muscle cells and the proliferation of endothelial cells, increase in tissue expression of ET-1 might lead to an increase in the tone of vasa vasorum and a concomitant decrease in vasa vasorum perfusion (33). Subsequently, hypoxia might develop within the vascular wall, constituting a potent stimulus for VEGF release, which might be further enhanced by additional stimulation of ET-1 expression (25,34). Indeed, in the same model of experimental hypercholesterolemia used in this study, development of endothelial dysfunction has been reported in the coronary circulation on the level of both the macrovasculature and the microvasculature and has been attributed, at least in part, to the endogenous endothelin system due to the fact that chronic administration of ET-A antagonist preserves endothelial function on both these levels (10,11). Recent reports confirm that vasa vasorum are sensitive to various vasoactive substances, among which ET-1 exerts a very strong vasoconstrictive effect (35). Thus, in line with these findings it may be speculated that attenuation of coronary neovascularization by chronic endothelin receptor antagonism may be secondary to preservation of endothelial function and improvement of vascular perfusion.

Coronary neovascularization in experimental hypercholesterolemia. In a primate model, progression of experimental hypercholesterolemia has been shown to be associated with a marked increase in blood flow to the media of coronary arteries as well as with a marked increase in flow during adenosine-induced vasodilation (36). Conversely, regression of hypercholesterolemia was associated with a reversal of these alterations (37). Although formation of new vasa vasorum rather than dilation of existing vessels was suggested and a cause-and-effect relation with experimental hypercholesterolemia was established on the basis of these findings, further insight into the mechanisms underlying these proliferative changes was pending. It has been suggested that neovascularization of coronary vasa vasorum might be triggered by structural alterations of the vascular wall due to increase in lipid load and vessel thickness (14). Indeed, experimental findings on the reduction in plaque growth by inhibition of plaque neovascularization seem to confirm an important role of vasa vasorum neovascularization for afferent and efferent nutrient supply as well as for the structural integrity of the arterial wall in the atherosclerotic and pre-atherosclerotic disease state (38). However, in a recent study, we found that coronary vasa vasorum neovascularization precedes, rather than follows, atherosclerotic lesion formation (15). Taking these, and the current, results into consideration, an increase in spatial density of coronary vasa vasorum in experimental hypercholesterolemia seems to result from active stimulation of neovascularization rather than from passive adaptation to structural alterations within the vascular wall. In this respect, the increase in the vascular tissue expression of ET-1 in experimental hypercholesterolemia might be of dual importance. For, not only by increase in vascular tone of vasa vasorum and subsequent decrease in vasa vasorum perfusion and hypoxia within the vascular wall, but also by direct stimulation of vascular smooth muscle cells, ET-1 can trigger the release of potent angiogenic factors such as VEGF within the vascular wall. Thus, experimental hypercholesterolemia seems to provide an active stimulus for neovascularization of coronary vasa vasorum with at least partial involvement of the endogenous endothelin system, rather than leading to vasa vasorum proliferation as an adaptive response secondary to structural alterations of the coronary arterial wall.

Study limitations. Although the definite nature of the underlying stimuli and mechanisms for vasa vasorum neovascularization in experimental hypercholesterolemia remains to be determined, this study identifies the endogenous endothelin system as an important pathophysiologic element. Even if the potential temporary impact of hemodynamics cannot be fully excluded due to lack of continuous registration of hemodynamic parameters, we have previously demonstrated no significance difference in hemodynamic parameters between animals with and without chronic ET-A antagonism in addition to a high cholesterol diet (11). Furthermore, this study focused on the coronary circulation, limiting the implications for other vascular beds.

Conclusions. This study, for the first time, demonstrates that chronic endothelin receptor antagonism prevents the increase in VEGF expression in the coronary arterial wall as well as the increase in the spatial density of coronary vasa vasorum in experimental hypercholesterolemia. Thus, this study supports a role for the endogenous endothelin system in coronary vasa vasorum neovascularization in pathophysiologic disease states, such as hypercholesterolemia.

Reprint requests and correspondence: Dr. Amir Lerman, Division of Cardiovascular Diseases, Mayo Clinic Rochester, 200 First Street Southwest, Rochester, Minnesota 55905. E-mail: lerma. amir@mayo.edu.
REFERENCES


