

Coronary vasa vasorum neovascularization precedes epicardial endothelial dysfunction in experimental hypercholesterolemia

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Abstract

Objective: Experimental hypercholesterolemia is associated with vasa vasorum neovascularization, unknown to occur before or after initial lesion formation. Thus, this study was performed to determine the temporal course of neovascularization of coronary vasa vasorum in relation to endothelial dysfunction, a hallmark of early atherosclerosis. **Methods:** Female domestic pigs were fed a normal diet (Group 1), a hypercholesterolemic diet for 2 and 4 weeks (Group 2), or a hypercholesterolemic diet for 6 and 12 weeks (Group 3). In vitro analysis of relaxation response to bradykinin served as an index for epicardial endothelial function. Spatial pattern and density of coronary vasa vasorum were assessed by three-dimensional microscopic computed tomography. **Results:** Relaxation response of coronary arteries to bradykinin was normal in both Group 1 ($93 \pm 6\%$) and Group 2 ($89 \pm 7\%$) but impaired in Group 3 ($71 \pm 11\%$; $P < 0.05$ vs. Group 1 and 2). In contrast, density of coronary vasa vasorum was significantly higher in both Group 2 (4.88 ± 2.45 per-mm²) and Group 3 (4.50 ± 1.37 per-mm²) compared to Group 1 (2.97 ± 1.37 per-mm²; $P < 0.05$ vs. Group 2 and 3). **Conclusion:** This study demonstrates that coronary vasa vasorum neovascularization occurs within the first weeks of experimental hypercholesterolemia and prior to the development of endothelial dysfunction of the host vessel, suggesting a role for vasa vasorum neovascularization in the initial stage of atherosclerotic vascular disease. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The development of atherosclerosis involves structural and functional changes in the vascular wall [1]. While endothelial dysfunction, characterized by impaired endothelium-dependent relaxation, is considered one of the earliest functional abnormalities in atherosclerosis, structural changes in the adventitia and specifically of the vasa vasorum may also take place early in the disease process [2–4].

Using a novel imaging technique, we have recently demonstrated increase in vasa vasorum spatial density of

porcine coronary arteries following 12 weeks of experimental hypercholesterolemia, which is also characterized by endothelial dysfunction and intimal thickening [5–8]. If these changes in the spatial structure of vasa vasorum precede the development of functional and structural changes of the host vessel and may therefore be involved in the initial stage of the atherosclerotic disease process, however, remained to be determined. Thus, the current study was designed to investigate the development of changes in the spatial structure of coronary vasa vasorum by high-resolution, three-dimensional computed tomography (micro-CT) in its temporal relationship to epicardial endothelial dysfunction in a porcine model of experimental hypercholesterolemia.

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2. Methods

2.1. Animals

The study has been approved by the Mayo Foundation Institutional Animal Care and Use Committee. In accordance with previous studies, female juvenile domestic pigs, weight 25–35 kg, were placed on a normal chow diet for 12 weeks (Group 1; $n=3$) or on a hypercholesterolemic diet (2% cholesterol, 15% lard, TD 93296, Harlan Teklad, Madison, Wisconsin) for 2 and 4 weeks (Group 2; $n=4$) or 6 and 12 weeks (Group 3; $n=4$) [5,7,8]. After completion of diet, blood samples were taken for analysis of plasma lipid levels [5]. Next, the animals were euthanized, the hearts immediately harvested and placed into a cold modified Krebs–Ringer bicarbonate solution [7].

2.2. In vitro analysis of vascular reactivity

Analysis of endothelium-dependent relaxation of coronary arteries has been previously described by our group [5]. Briefly, proximal coronary artery segment rings were mounted on a calibrated isometric force transducer and suspended in an organ chamber. Vascular viability was confirmed by the contractile response to KCL. At 6 g, all vessels were subsequently exposed to substance P (10^{-6} mol/l, Sigma, St. Louis, MO, USA) to verify the functional integrity of the vascular endothelium. Subsequently, epicardial arteries were contracted with 10^{-7} mol/l endothelin-1 (ET-1) and then relaxed with cumulative concentrations of 10^{-11} – 10^{-6} mol/l either bradykinin or calcium ionophore (Sigma, St. Louis, MO, USA). At the end of the experiment, $10^{-3.5}$ mol/l of papaverine (Sigma, St. Louis, MO, USA) was added to verify that rings maintained vasodilating capacity.

2.3. Micro-CT imaging

The left anterior descending coronary artery was prepared and scanned by micro-CT as described in detail by our group before [6,7,9,10]. The imaging system yielded an average number of 500 slices per coronary artery segment with a matrix of 42 μm cubic voxels and a 16-bits gray scale [6]. The resulting three-dimensional digitalized image was analyzed by using the Analyze[®] software package (version 3.1, Biomedical Imaging Resource, Mayo Foundation, Rochester, MN, USA) [7].

On average, 7–12 topographic cross-sections at 1 mm intervals along the segment were chosen for region of interest analysis, excluding potential sources of error such as branching points. The area of vasa vasorum analysis was determined as reported before and designated vessel wall area [7,9,11]. 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-

mm^2 vessel wall area), mean diameter of 1st and 2nd order vasa vasorum, and ratio of the number of 1st to 2nd 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 1st order vasa vasorum and ran circumferentially around the lumen [7].

2.4. Statistical analysis

All continuous data are expressed as mean \pm standard error (S.E.). Multiple group comparison was based on analysis of variance (ANOVA) with subsequent post hoc analysis for parametric and non-parametric data. Comparison of two groups was performed by either a paired or unpaired Student's *t*-test (parametric data) or a Mann–Whitney *U*-test (non-parametric data). A Fisher's exact test was applied for comparison of two groups with categorical variables. Statistical significance was accepted for $P<0.05$.

3. Results

3.1. Lipid profile

Compared to animals on a normal diet, animals on a high-cholesterol diet for 2 and 4 weeks and 6 and 12 weeks had a higher plasma concentration of total cholesterol (106 ± 6 , 145 ± 16 , and 309 ± 49 mg/dl, respectively, $P<0.01$) and LDL cholesterol (34 ± 12 , 83 ± 13 , 206 ± 92 mg/dl, respectively, $P<0.01$) without a significant increase in body weight.

3.2. Vascular reactivity

Endothelium-dependent relaxation of epicardial coronary arteries in animals fed with a high-cholesterol diet for 2 and 4 weeks was similar to normal animals (Fig. 1). However, the vasorelaxation response to bradykinin was significantly attenuated in pigs on a hypercholesterolemic diet for 6 and 12 weeks as compared to the other two groups of animals (Fig. 1). Pre-contraction response to ET-1 as well as relaxation response to calcium ionophore and papaverine were similar among all groups.

3.3. Vasa vasorum

There was a significant increase in both vasa vasorum density and vessel wall area in Group 2 compared to Group 1 (Table 1, Fig. 2). This increase was associated with a decrease in the mean diameter of the 1st order vasa vasorum. In Group 3, vessel wall area continued to increase while the number of vasa vasorum, vasa vasorum density, and diameter of the 1st and 2nd vasa vasorum were similar to Group 2.

As illustrated in Fig. 3, the spatial architecture of vasa

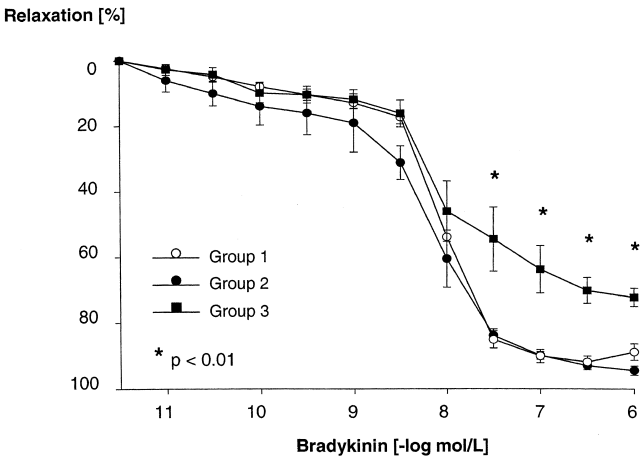


Fig. 1. Endothelium-dependent vasorelaxation of coronary arteries in response to increasing concentrations of bradykinin after pre-contraction with 10^{-7} mol/l endothelin-1. Compared to normal pigs (Group 1) and those on a high-cholesterol diet for 2 and 4 weeks (Group 2), significant impairment of EDR is seen in animals on a high-cholesterol diet for 6 and 12 weeks (Group 3).

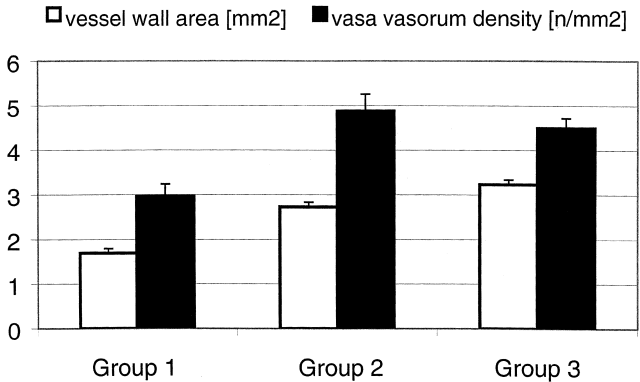


Fig. 2. Bar graph demonstrating vessel wall area (mm^2 ; blank columns) and vasa vasorum density (number per- mm^2 ; filled columns) in coronary arteries of normal animals (Group 1) and animals fed a high-cholesterol diet either for 2 and 4 weeks (Group 2) or for 6 and 12 weeks (Group 3). While there is a gradual increase in vessel wall area during progression of hypercholesterolemia ($P<0.001$ for all comparisons), increase in vasa vasorum density is established within 4 weeks after initiation of high-cholesterol diet ($P<0.05$ for Group 2 and Group 3 versus Group 1). Values are mean \pm standard error.

vasorum in animals on a normal diet was clearly structured into 1st and 2nd order vasa vasorum. In animals on high-cholesterol diet for 2 and 4 weeks a plexus of newly formed vasa vasorum becomes apparent. In animals on high-cholesterol diet for 6 and 12 weeks vasa vasorum structure appears to be even more complex and disorientated in association with the significantly increased ratio of 2nd to 1st order vasa vasorum.

4. Discussion

The current study demonstrates that an increase in coronary vasa vasorum density precedes the impairment of endothelial function of the host vessel. The temporal link may suggest a role for vasa vasorum neovascularization in the early stage of coronary atherosclerosis.

Several human atherectomy and autopsy series described plaque neovascularization in coronary atherosclerosis [4,12]. Although a correlation between neovascularization and extent of atherosclerosis has been reported in these studies, uncertainty remained concerning the initiation of this process.

In a functional approach, a marked increase in blood flow through coronary vasa vasorum both at baseline and after injection of adenosine was found in hypercholesterolemic monkeys compared to normal controls [13]. Formation of new vessels rather than dilation of existing vasa vasorum was suggested on the basis of these findings, obtained from epicardial vessels with initial to advanced types of atherosclerotic plaques [13]. Using high-resolution micro-CT, we were recently able to confirm the putative increase in coronary vasa vasorum density in experimental hypercholesterolemia and to visualize it as a dense plexus of finely anastomosing microvessels, primarily 2nd order vasa vasorum, with disorganized spatial appearance [7]. The current study extends these previous observations by demonstrating the development of these structural alterations within 4 weeks of high-cholesterol diet and prior to vasofunctional alteration of the host vessel. Segmental differences in vasoreactivity and vasa vasorum density cannot be excluded by means of the study methods used and must therefore be considered as a potential study limitation for the interpretation of the study data. Due to the systematic nature of this potential error, however, all animal groups are affected to the same extent, allowing

Table 1
Micro-CT parameters

	Group 1 (n=26)	Group 2 (n=41)	Group 3 (n=39)	P value
Vessel wall area (mm^2)	1.69 ± 0.08	2.73 ± 0.09	3.23 ± 0.11	$P<0.001$ for all
Vasa vasorum count (n)	4.73 ± 0.34	$13.98 \pm 1.22^*$	$14.31 \pm 0.72^*$	$*P<0.05$ vs. Group 1
Vasa vasorum density (n/mm^2)	2.97 ± 0.27	$4.88 \pm 0.38^*$	$4.50 \pm 0.22^*$	$*P<0.05$ vs. Group 1
Ratio 2nd/1st order vasa vasorum	1.83 ± 0.26	$4.06 \pm 0.46^*$	$4.96 \pm 0.44^*$	$*P<0.05$ vs. Group 1
Diameter 1st order vasa vasorum	121.08 ± 8.45	101.67 ± 2.34	$96.46 \pm 3.66^*$	$*P<0.05$ vs. Group 1
Diameter 2nd order vasa vasorum	57.22 ± 2.11	58.08 ± 1.26	53.83 ± 0.98	NS

Values are mean \pm S.E. per number of cross-sections analyzed (n).

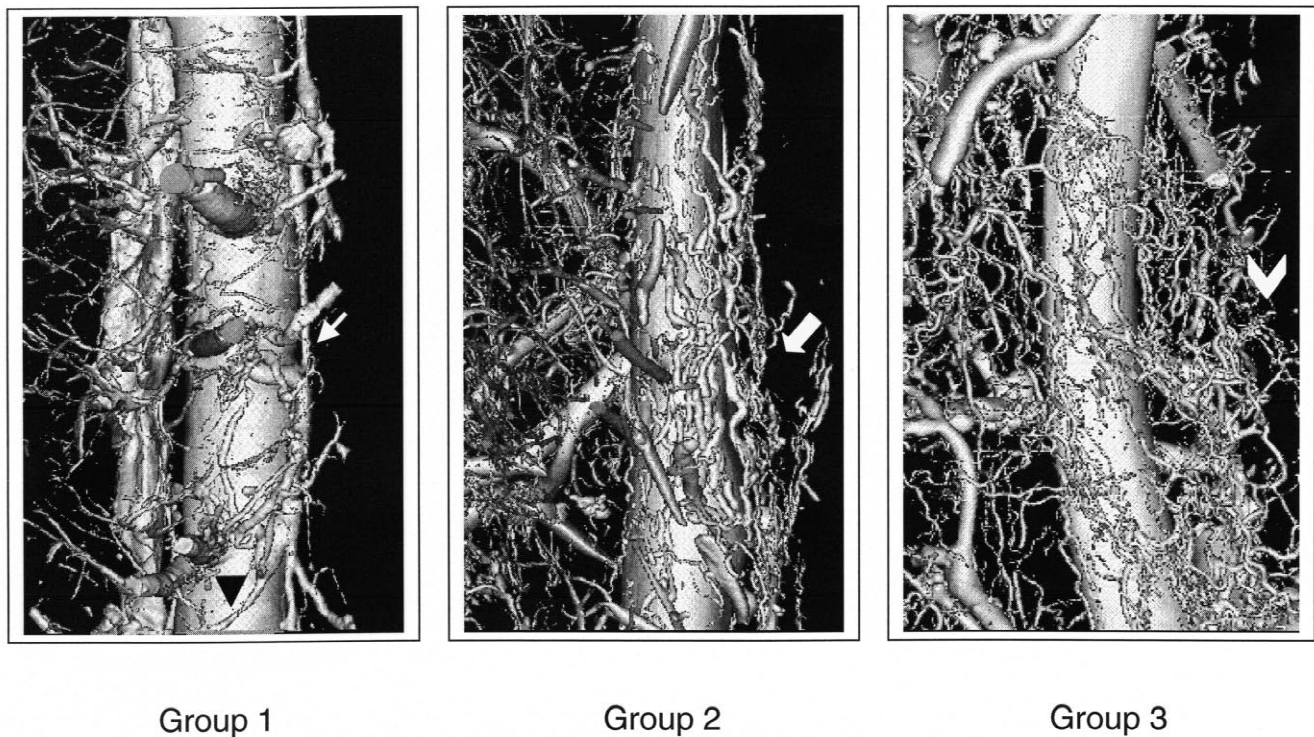


Fig. 3. Microscopic computed tomography images of the left anterior descending artery from normal animals (Group 1; left panel) and animals fed a hypercholesterolemic diet either for 2 and 4 weeks (Group 2; middle panel) or for 6 and 12 weeks (Group 3; right panel). In Group 1 the spatial pattern of vasa vasorum is characterized by a clear separation into 1st order vasa vasorum, running longitudinally (small arrow), and 2nd order vasa vasorum, running circumferentially (arrow head). In both Group 2 and Group 3 a network of newly formed vasa vasorum surrounds the host vessel (arrow), predominantly formed by 2nd order vasa vasorum (open arrow). Reconstruction voxel for illustration 21 μm .

group-wise comparison in regard to the temporal course. Thus, vasa vasorum neovascularization may be regarded as a phenomenon of the pre-atherosclerotic disease state although it has to be considered that bradykinin-dependent vasodilation, as used in the current study, may not represent the whole spectrum of endothelial cell function.

Owing to the correlation between vasa vasorum neovascularization and vessel thickness, insufficiency of the existing diffusional network to uphold nutrient and oxygen supply to a vascular wall, increasing in size, has been suggested as a causal mechanism [7]. Accordingly, tissue hypoxia could lead to vasa vasorum neovascularization by stimulation of the production of angiogenic factors such as vascular endothelial growth factor (VEGF) [15]. Functional impairment of vasa vasorum perfusion due to increase in vasa vasorum tone might further contribute to the development of local hypoxia and might render the host vessel as susceptible to atherosclerotic changes as structural obstruction of vasa vasorum perfusion [16,17]. Indeed, decrease in diameter of 1st order vasa vasorum during experimental hypercholesterolemia has been found in the current study as well as in our previous study and might be a reflection of these functional alterations [7]. Furthermore, similar findings of increase in vessel density with overall reduction of vessel diameter have recently been reported for myocardial microvessels in hypercholes-

terolemic pigs as has been the development of endothelial dysfunction of these vessels in experimental hypercholesterolemia [5,10]. Thus, increase in vascular tone of vasa vasorum microvessels and reduction in nutrient supply to the coronary artery wall might lead to a compensatory increase in vasa vasorum density in experimental hypercholesterolemia.

Another important aspect, noted in human histomorphologic studies before, is the relationship between neovascularization and the extent of inflammatory cell infiltration [12]. In giant cell arteritis, a close relationship between inflammation, VEGF production and vasa vasorum neovascularization has been shown [18]. As both inflammation and stimulation of the expression of angiogenic factors are also found in atherosclerotic lesions, vasa vasorum neovascularization might alternatively be part of an inflammatory response mechanism to vascular injury [1,19,20].

Initial reports characterized newly formed vasa vasorum as improper, fragile vessels which might favor complication of atherosclerotic lesions such as hemorrhage and rupture [4,20]. Yet, there might also be a role for neovascularization in plaque initiation and progression as an expanding vascular network might increase transmural flux of plasma nutrients and tissue inflammation [7,14]. Indeed, in recent experimental study with apolipoprotein E-de-

ficient mice it has been demonstrated that inhibition of progression of neovascularization resulted in a marked reduction in plaque growth [21]. Thus, neovascularization might contribute not only to complication of atherosclerosis but also to initiation and progression of the atherosclerotic disease process.

Another finding of the current study is the change in the relationship of 2nd to 1st order vasa vasorum during experimental hypercholesterolemia, indicating more extensive increase in 2nd than 1st order vasa vasorum rather than multiplication of the existing network. Notably, these plexus of newly formed vasa vasorum are focally enhanced in areas of increased vessel wall thickness and atherosclerotic lesion formation [4]. Thus, vasa vasorum neovascularization has been related predominantly to sites of atherosclerotic lesion formation in previous studies and has been shown to precede them in the current study and might therefore play a role in the initial stage of atherosclerosis.

5. Conclusion

The current study demonstrates that coronary vasa vasorum neovascularization occurs within the first 4 weeks experimental hypercholesterolemia and prior to the development of endothelial dysfunction, a hallmark of the early disease state. The temporal link may suggest a role for vasa vasorum neovascularization in the initial stage of coronary artery disease.

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