Coexistence of Hypercholesterolemia and Hypertension Impairs Adventitial Vascularization

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Abstract—We have shown that adventitial vasa vasorum (AVV) formation is enhanced in hypertensive rat aorta to compensate hypoxia in the thickened media and that hypercholesterolemia impairs angiogenesis in rat ischemic hindlimb. Thus, we examined the effects of coexistence of hypercholesterolemia and hypertension on AVV formation. In Wistar rats, hypercholesterolemia was established by high-cholesterol diet from Day −14 (HC rats), and hypertension was induced by a suprarenal aortic constriction at Day 0 (HT rats). At Day 28, we studied AVV density, adventitial area, and medial thickness in the ascending aorta of control (standard diet + sham operation), HC, HT, and HC+HT rats (n=5/group). In HC rats, although the adventitial area was modestly increased, the AVV density and medial thickness were unchanged versus controls. In addition to medial thickening, marked enlargement of the adventitial area accompanied by increased AVV density was observed in HT rats, compared with controls. HC+HT rats showed lower AVV density, despite larger adventitial area, than HT rats, whereas the medial thickness was similar in HT and HC+HT rats. Immunohistostaining revealed hypoxia-inducible factor-1α expression in the media only in HC+HT rats but not in the other 3 groups, suggesting persistent medial hypoxia in HC+HT rats. In conclusion, it is suggested that coexistence of hypercholesterolemia and hypertension impairs AVV formation, resulting in insufficient compensation for hypoxia in the thickened media. Our findings provide an insight into the mechanism of the aggravation of arteriosclerosis when both hypercholesterolemia and hypertension are present.

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Key Words: vasa vasorum ■ hypercholesterolemia ■ hypertension, essential ■ arteriosclerosis ■ hypoxia

The arterial wall can receive oxygen and nutrients by diffusion outwards from the main lumen and inwards from adventitial vasa vasorum (AVV) in the arteries in which the media does not exceed the “critical depth” (ie, <500 μm in the thickness or <29 lamellar units), which are typically seen in most of conduit and muscular arteries of large animals, including dogs, monkeys, and humans, and in the arteries of small animals such as rat aorta. In contrast, in the arteries in which medial thickness exceeds the “critical depth,” vasa vasorum originating from AVV are found in the media and contribute to the oxygen supply in the outer media. Interference of AVV flow by occlusion of the AVV with thrombin-induced thrombus or by ligating the vessels that supply the AVV results in medial necrosis and intimal thickening in dogs. Also, the arterial wall oxygen supply is impaired after balloon injury but later compensated by the formation of new AVV. Therefore, the role of AVV has been highlighted in vascular wall homeostasis to maintain normal structure and function of the arteries, whereas intimal/medial vasa vasorum may contribute to growth of the atherosclerotic plaque and plaque rupture by nourishing expanding plaque and by delivering inflammatory cells. However, little attention was paid to changes in AVV during arteriosclerotic vascular remodeling.

Hypercholesterolemia (HC) and hypertension (HT) each is a major risk factor for arteriosclerosis and the presence of both conditions accelerates arteriosclerosis. It has been shown that hypercholesterolemia increases AVV formation in the monkey coronary artery. And, our recent study has demonstrated that hypertension induces proliferation of the endothelial cells (ECs) in the adventitia and subsequently AVV formation in the rat aorta to compensate for hypoxia in the thickened media during hypertensive remodeling. It is possible that the alternations in AVV formation may be involved in the pathogenesis of aggravation of the arteriosclerotic changes when both hypercholesterolemia and hypertension are present. However, little is known regarding the effects of coexistence of these risk factors on AVV formation. Accordingly, in the present study, we sought to examine the effects of hypercholesterolemia on AVV formation in hypertensive rat aorta.

Methods

Animal Model

All procedures were approved by the Institutional Animal Care and Use Committee. Male Wistar rats (300 to 350 g) were fed with...
standard diet or 2% high-cholesterol diet from Days −14 to 28. At Day 0, rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg), and were randomized to undergo a supraparenal abdominal aortic constriction or the sham operation. This study included the following 4 groups: control rats with standard diet + sham operation, HC rats with high-cholesterol diet + sham operation, HT rats with standard diet + aortic constriction; and HC+HT rats with high-cholesterol diet + aortic constriction. Unless otherwise indicated, 5 rats were included in each group. Blood pressure was measured in rats in an unrestrained conscious state through a heparinized polyethylene catheter indwelled into the left carotid artery. Serum total cholesterol level was determined enzymatically with a commercially available kit (Wako Chemicals).

### Morphometry and Immunohistostaining

At Day 28, rats were killed with an overdose injection of pentobarbital and then perfusion-fixed with 4% glutaraldehyde in Hanks’ solution at 100 mm Hg. The ascending aorta was excised and processed for histological morphometry and immunohistostaing study as previously described. For morphometry, 4 independent Mallory-Azan stain sections of each rat were scanned and analyzed. Nonround vessels, because of oblique transsection, were excluded.

The adventitial area was defined as the area enclosed by the outer border of the external elastic lamina and the outer border of the area of dense fibrous tissues immediately surrounding the media. The medial thickness is defined as the minimal distance between the outer border of the internal and external elastic laminae. Immunohisto-stainings for hypoxia-inducible factor (HIF)-1α and von Willebrand factor (vWF) were performed as described elsewhere. The AVV was defined as the luminal structure surrounded by a layer of vWF-labeled ECs with or without medial smooth muscle cell (SMC) layer in the adventitia. The AVV density was determined as the ratio of AVV count to adventitial area. Polyclonal goat antibody for HIF-1α and monoclonal mouse antibody for vWF were purchased from Santa Cruz Biotech and Dako, respectively. Each quantitative analysis was done in 4 independent whole sections (×200 magnification) of each rat by a single observer in a blind manner.

### Immunoblotting Study

After extraction from the homogenates of the unfixed aorta (n=3/group), tissue protein was separated by 12% SDS-PAGE and subjected to immunoblotting for HIF-1α, as previously described. The signals were detected and analyzed using the chemiluminescence detection system (Amersham Pharmacia Biotech).

### Statistical Analysis

One-way ANOVA followed by Scheffe’s F test was performed for the statistical comparisons. A value of *P*<0.05 was considered statistically significant.

### Results

The serum cholesterol level was significantly higher in high cholesterol-fed than in normal-fed rats at Day 28 (Figure 1A). Arterial pressure was not affected by the types of diet (Figure 1B). At Day 28, control and HC rats showed arterial pressure levels similar to that of the intact rats (control rats at Day 0). Aortic constriction induced a rapid and significant rise in arterial pressure, and thereafter hypertension sustained in HT rats, as described previously. There was no significant difference in arterial pressure levels between HT and HC+HT rats.

### Medial Thickening

The medial thickness did not differ among the 4 study groups at Day 0. At Day 28, there was no significant difference in medial thickness between control and HC rats (Figures 2 and 3A). Hypertension induced medial thickening, and the medial thickness in HT rats was increased by 1.6-fold of control rats. HC+HT rats showed medial thickness similar to HT rats (1.7-fold of controls).

### AVV Formation and Adventitial Thickening

A small number of AVV were observed in thin, fibrous adventitia in the intact rats. There were no significant differences in the AVV density and adventitial area among the 4 groups at Day 0 (data not shown). Effects of hypercholesterolemia and hypertension were investigated at Day 28 (Figures 2 and 3). In control rats, the AVV density and adventitial area were not changed compared with the intact rats. HT rats exhibited marked increase in the adventitial area (3.2-fold of controls), and various sized AVV were scattered in the thickened adventitia with the AVV density of 2.2-fold of controls. In HC rats, the adventitial area was increased by 2.6-fold of controls, but the AVV density was unchanged compared with that in controls. HC+HT rats had further increased adventitial area (5.7-fold of controls) compared with HT rats. However, the AVV density was significantly smaller in HC+HT (1.7-fold of controls) than HT rats. On the basis of light microscopic examination, there were no appar-
ent differences in the morphology and the distribution of newly formed AVV among HC, HT, and HC+HT rats. In addition, the vasa-like structure was not found in the media or intima in the 4 groups. No apparent intimal thickening was observed in any study groups at Day 28.

**HIF-1α Expressions**

Recently, we demonstrated that expression of HIF-1α, an inducible factor in hypoxic cells, is not detected in the aortic wall of the intact rats and is transiently induced in medial SMCs in HT rats with a peak at Day 7, declining to undetectable levels at Day 28. Consistent with the previous observations, immunoblotting analysis showed that HIF-1α was not significantly expressed in the aortic wall in control and HT rats at Day 28 (Figure 4A). Although there was no apparent HIF-1α expression in HC rats, HC+HT rats showed significant HIF-1α expression at Day 28. Immunohistochemistry revealed that in HC+HT rats, the nuclei of SMCs in the outer layers of the media were labeled with HIF-1α (Figure 4B).

**Discussion**

The new findings of this study are as follows: (1) although hypertension increased the AVV density associated with medial thickening in normocholesterolemic rats, the hypertension-induced augmentation of the AVV density was reduced by hypercholesterolemia without significant effects on the medial thickening, and (2) HIF-1α expression was observed in the outer layers of medial SMCs only in HC+HT rats, but not in control, HC, or HT rats, at Day 28.

In the present study, medial thickness did not exceed the “critical depth” in any groups. Consistent with earlier studies, vasa vasorum formation was not found in the media and intima in not only control but also HC, HT, and HC+HT rats by Day 28. Therefore, aortic remodeling of rats with the aortic constriction is a model of arteriosclerosis of the arteries that have no medial/intimal vasa vasorum but is not an adequate model of atherosclerotic remodeling of the arteries with the medial thickness exceeding the “critical depth” that have medial vasa, which is typically seen in the aorta and the coronary and femoral arteries of the large animals.

The count of AVV may depend on the adventitial area. Thus, to avoid the possible effects of adventitial area, we adopted the AVV density (the ratio of the AVV count over adventitial area) as an indicator for AVV formation rather than the AVV count.

**Hypertension and AVV Formation**

An earlier study demonstrated that increased thickness of the arterial wall caused by hypertension increases the distance required for oxygen diffusion from the lumen and subsequently produces a relatively hypoxic state in the outer to middle layers of the media. Recently, we have demonstrated in rat aorta that (1) hypertension induces adventitial EC proliferation and resultant AVV vascularization, concomitant with medial and adventitial thickening, (2) the adventitial EC proliferation is associated with transient induction of HIF-1α and vascular endothelial growth factor (VEGF) in the outer layers of medial SMCs after Day 3 with a peak at Day 7 when medial thickening becomes evident, and (3) the HIF-1α and VEGF expressions returned to insignificant levels by Day 28, although further medial thickening was observed. Thus, it is suggested that AVV formation is a compensatory mechanism for hypoxia in the thickened media during hypertensive remodeling via the HIF/VEGF-mediated pathway and that medial hypoxia is compensated for at Day 28 by oxygen supply through the augmented AVV formation. The results consistent with these observations were obtained in HT rats as shown in Figures 2 to 4.

**Coexistence of Hypercholesterolemia and Hypertension**

The most important finding of this study is that HC+HT rats showed the AVV density lower than HT rats, whereas the medial thickness did not significantly differ between the 2 groups (Figures 3 and 4). In HC rats, the AVV density was

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**Figure 4.** Representative microphotographs of immunoblotting (A) and immunohistochemistry (B) for HIF-1α at Day 28. The similar results were obtained from 3 and 5 independent experiments in panels A and B, respectively. Control, standard diet+sham operation; HC, high-cholesterol diet+sham operation; HT, standard diet+aortic constriction; HC+HT, high-cholesterol diet+aortic constriction. Arrows indicate the HIF-1α-labeled nuclei.

**Figure 3.** Effects of hypertension and hypercholesterolemia on medial thickness (A), adventitial area (B), and adventitial vasa vasorum density (C) in rat aorta. Control, standard diet+sham operation; HC, high-cholesterol diet+sham operation; HT, standard diet+aortic constriction; HC+HT, high-cholesterol diet+aortic constriction. Bar=1×SD (n=5). *P<0.05 and **P<0.01 versus Control. †P<0.05 and ††P<0.01 versus HC. δP<0.05 and δδP<0.01 versus HT.
similar to that of controls. Thus, it is suggested that hypertension-induced AVV formation is specifically reduced by the copresence of hypercholesterolemia. Furthermore, it is noteworthy that HIF-1α expression was observed in medial SMCs only in HC+HT rats but not in HT rats (Figure 4), suggesting the presence of medial hypoxia, because HIF-1α is a sensitive marker for tissue hypoxia.20 Accordingly, the oxygen supply through the increased AVV may be insufficient to compensate for medial hypoxia in HC+HT rats at Day 28. Because the medial thickness was similar in HT and HC+HT rats, the persistent hypoxia in the media of HC+HT rats may be attributable to lower AVV density than that in HT rats.

In the present study, the mechanism whereby hypercholesterolemia impairs AVV formation specifically in hypertensive rats was not elucidated. In this study, despite the persistent expression of HIF-1α, AVV formation was impaired in HC+HT rats. These observations may raise the possibility that coexistence of hypercholesterolemia and hypertension inhibits the HIF-1α-mediated production of angiogenic factors and/or deteriorates the efficiency of the angiogenic factors in the rat aorta. There is increasing evidence that nitric oxide acts as a critical modulator for angiogenesis in hypoxic tissue.20,21 Recently, we and others have demonstrated that hypercholesterolemia markedly attenuates angiogenesis in response to hindlimb ischemia in rats.22,23 And, we have shown that reduced bioactivity of nitric oxide in hypoxic tissue is related to the impaired angiogenesis.23 However, hypercholesterolemia itself did not impair AVV formation in our rat model. It is also well known that nitric oxide bioactivity is impaired in hypertension. Thus, decreased activity of the nitric oxide-mediated pathway may be involved in the mechanism of impaired AVV formation in HC+HT rats. However, it is possible that other undetermined factors are involved in the mechanism whereby AVV formation was attenuated in HC+HT rats, but not in HC rats. The precise molecular mechanisms for impaired AVV formation during hypertensive remodeling in hypercholesterolemic animals should be determined in future studies.

In addition, hypercholesterolemia impairs the EC function via the nitric oxide-dependent and -independent manners. Thus, it is plausible that hypercholesterolemia deteriorates AVV function by impairing the EC function of not only the AVV but also the feeding arteries for the AVV. And, in the feeding arteries that are directly exposed to high blood pressure, further EC damages may be induced by coexistence of hypercholesterolemia and hypertension. Accordingly, it is conceivable that impaired EC function in the AVV and the feeding arteries results in further reduction in the blood flow through the AVV.

Persistent medial hypoxia induced by coexistence of hypercholesterolemia and hypertension may aggravate arteriosclerotic processes. It is likely that medial hypoxia directly impairs SMC function and induces necrosis of the medial SMCs.4,5 Also, it has been documented that various growth factors and cytokines that are involved in the migration and proliferation of vascular cells, such as transforming growth factor-β, platelet-derived growth factor, endothelin, and VEGF, are upregulated under conditions of low oxygen tension.24-28 In addition, a decrease in the oxygen tension would result in incomplete oxidation probably leading to increased concentrations of free radicals and abnormalities of the redox state.29 Oxidative stress may, in turn, activate gene expression involved in generating an inflammatory response in the arterial wall.10 Taken together, persistent medial hypoxia associated with impaired AVV formation may lead to further vascular damage and arteriosclerotic changes. Thus, future studies should determine the effects of hypercholesterolemia and hypertension on vasa vasorum formation in the chronic phase.

Study Limitations

The suprarenal aortic constriction model is a model of acute hypertension. Thus, there is a limitation to extrapolate the results of this study to the mechanisms of vascular remodeling and AVV formation during chronic hypertension. Also, the potential remodeling of the vascular wall proximal to the constriction may be involved in AVV formation observed in this model. Second, blood flow through vasa vasorum was not measured in the present study. Thus, it remains undetermined if blood flow within the aortic wall was correlated with the AVV density. Third, it remains undetermined which cholesterol (LDL, oxidized LDL, or HDL) plays an important role in the observed effects of hypercholesterolemia on AVV formation in the present study. Because oxidized LDL, but not normal LDL, has been shown to inhibit EC growth and angiogenesis ex vivo,30 it is possible that not only the alterations in the serum cholesterol profile but also the changes in the oxidation levels of cholesterol importantly affect AVV formation. Thus, this issue should be addressed in future studies. In addition, it has been shown that in a transgenic model of ischemic cardiomyopathy, the metalloelastase-positive monocytes/macrophages drill well-organized tunnel-like structures, usually void of ECs, in ischemic myocardium, suggesting that the inflammatory cells participate in the elaboration of compensatory neovascularization.31 It remains unknown whether the novel mechanism of neovascularization is involved in AVV formation in response to hypertension. Finally, a very recent study has shown that growth factors induce HIF-1α expression through the Akt-mediated pathway in some cell types other than SMCs.32 We cannot deny the possibility that this mechanism is involved in HIF-1α expression in HC+HT rats.

In conclusion, coexistence of hypercholesterolemia and hypertension impairs AVV formation in the rat aorta. The impaired AVV formation may result in insufficient compensation for hypoxia in the thickened media in HC+HT rats. Our findings provide an insight into the mechanism whereby coexistence of major risk factors aggravates arteriosclerotic changes.

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