Hypoxia-Inducible Factor-1α/Vascular Endothelial Growth Factor Pathway for Adventitial Vasa Vasorum Formation in Hypertensive Rat Aorta

Fumitaka Kuwahara, Hisashi Kai, Keisuke Tokuda, Rei Shibata, Ken Kusaba, Nobuhiro Tahara, Hiroshi Niiyama, Tsuyoshi Nagata, Tsutomu Imaizumi

Abstract—The roles of adventitial vasa vasorum have been highlighted in vascular wall homeostasis. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor in physiological and pathophysiological conditions. However, little is known regarding the changes in adventitial vasa vasorum and the mechanism of the formation in hypertensive arteries. Accordingly, endothelial cell proliferation, adventitial vasa vasorum count, and expression of VEGF signaling axis proteins were examined in the ascending aorta of hypertensive Wistar rats that underwent suprarenal aortic constriction. Hypertension not only induced medial and adventitial thickening but also significantly increased adventitial vasa vasorum count by day 28. Preceding the medial thickening, BrdU\(^+\)-proliferative endothelial cells were observed in the adventitia but not in the media and intima after day 3; they peaked at day 7 and remained modestly increased at day 28. The BrdU\(^+\) endothelial cells showed induction of Ets-1, a transcription factor mediating angiogenic response of VEGF. Furthermore, concomitant expression of VEGF and a hypoxia-inducible transcription factor (HIF-1α) was observed in the outer layers of medial smooth muscle cells at day 3 and extended to the middle layers of medial smooth muscle cells at day 7, returning to lower levels by day 28. In conclusion, adventitial vasa vasorum formation was induced by hypertension through the HIF-1α/VEGF/Ets-1 pathway during hypertensive remodeling. (Hypertension. 2002;39:46-50.)

Key Words: vasa vasorum ● hypertension ● VEGF ● HIF-1α ● endothelial cells

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dventitial vasa vasorum (AVV) are important in maintaining normal structure and function of arteries, whereas medial/intimal vasa vasorum are implicated in the progression of atherosclerotic plaque and plaque rupture by nourishing expanding plaque and delivering inflammatory cells.\(^1,2\) There is convincing evidence that disruption of blood flow to the AVV results in medial necrosis\(^3\) and intimal hyperplasia.\(^4,5\) Moreover, it has been demonstrated that in balloon-injured arteries, the arterial wall oxygen supply is impaired after injury but is later compensated for by new AVV formation.\(^6\) Accordingly, the role of AVV has been highlighted in vascular wall homeostasis. Most conduit and muscular arteries have vasa vasorum in the adventitia but not in the media; oxygen and nutrients are supplied by diffusion from the AVV to the outer media and from the main vessel lumen to the inner media.\(^7\) Oxygen requirements of the vessel wall itself are relatively modest.\(^8\) However, medial thickening may create the hypoxic zone in the media by increasing the distance required for oxygen diffusion from the lumen.\(^9,10\) Because hypertension produces medial thickening, it is conceivable that the AVV plays a role in the maintenance of homeostasis during vascular remodeling in hypertensive arteries. However, there has been little study of the AVV in hypertensive arteries.

Vascular endothelial growth factor (VEGF) has potent mitogenic and promigratory actions specific for endothelial cells (ECs), intimately linked with new vessel development in physiological and various diseased situations.\(^11,12\) These stimulatory effects on ECs are initiated by the binding of VEGF to its high-affinity tyrosine kinase receptor Flk-1\(^13\) and subsequently result in activation of a transcription factor, Ets-1, leading to conversion of ECs to the angiogenic phenotype.\(^14,15\) A transcriptional regulator, hypoxia-inducible factor (HIF)-1α, plays a general role by signaling the existence of hypoxia to the transcriptional machinery in the nucleus of various cell kinds.\(^16\) Moreover, the role of HIF-1α in activation of VEGF gene transcription is known for hypoxic cells.\(^17\) Therefore, it is possible that the HIF-1α/VEGF/Ets-1 pathway is involved in the regulation of AVV formation in hypertensive artery.

Accordingly, we hypothesized that AVV increases during hypertensive vascular remodeling and the HIF-1α/VEGF/Ets-1 pathway is involved in the mechanism. Therefore, EC proliferation, AVV count, and expression of VEGF and related proteins were investigated in the ascending aorta of hypertensive rats that underwent constriction of the suprarenal abdominal aorta in the present study.
Methods

All procedures were in accordance with institutional guidelines of animal care and treatment. Male Wistar rats (8 weeks old, Japan SLC Inc) were anesthetized with sodium pentobarbital (50 mg/kg IP), and a suprarenal abdominal aorta constriction (AC) or the sham operation was established. Blood pressure was measured in rats in an unrestricted, conscious state through a heparinized polyethylene catheter indwelled into the left carotid artery. Unless otherwise indicated, 7 rats were studied in each group at each time point. Sources of antibodies were as follows: for polyclonal rabbit antibodies for VEGF (sc507) and Ets-1 (sc350), Santa Cruz Biotech; for polyclonal goat polyclonal antibody for HIF-1α (sc8711), Santa Cruz Biotech; and for monoclonal mouse antibodies for von Willebrand factor (vWF), α-smooth muscle actin (SMA), and vimentin, DAKO.

Tissue Preparation and Morphometry
Rats were killed with an overdose injection of sodium pentobarbital and then perfusion fixed with 4% glutaraldehyde in Hanks’ solution at 100 mm Hg. The ascending aorta was immediately excised, embedded in paraffin, and cut into 5-μm cross sections. For morphometry, 4 independent Mallory-Azan stain sections of each rat were scanned and analyzed with a digital image analyzer by a single observer in a blinded manner. 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. Vessels that were not round because of oblique transection were excluded.

Immunohistostaining and In Situ BrdU Labeling
The cross sections were subjected to immunohistochemistry with a denoted primary antibody and a commercially available detection system (LSAB2 kit for rabbit IgG, DAKO; VECTASTAIN for goat IgG, Vector laboratories) according to the manufacturer’s instructions. To identify proliferating cells, in situ BrdU labeling was performed with a cell proliferating kit (Amersham Pharmacia Biotech). Cell type showing BrdU incorporation was determined on the basis of double immunostaining against vWF, vimentin, and SMA for ECs, fibroblasts, and smooth muscle cells (SMCs), respectively, with the commercially available Double Immunostain Kit (DAKO). The AVV were defined as the luminal structure surrounded by a layer of vWF+ ECs in the adventitia. SMA+ cells surrounding the EC layer of the AVV were regarded as AVV SMCs. The positively stained cells and AVV were counted and averaged in 4 independent entire sections of each rat by an observer in a blinded manner.

Immunoblotting Study
After it was extracted from the homogenates of the unfixed aorta (n=4 in each group), tissue protein was separated by 12% SDS-PAGE and blotted onto polyvinylidene difluoride membrane. Blots were probed with a primary antibody for VEGF or HIF-1α, and the signals were detected and analyzed by use of the chemiluminescence detection system (Amersham Pharmacia Biotech) as previously described.

Statistical Analysis
Data are expressed as mean±SD. Student’s t test or 1-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

Hypertensive Aortic Remodeling
AC induced vascular remodeling in the rat aorta, associated with a rapid and significant rise in mean arterial pressure (Figure 1). In intact and sham rats, adventitia consisted of thin, fibrous layers. In AC rats, medial and adventitial thicknesses significantly increased after day 7, and medial thickness and adventitial area at day 28 reached 156% and 322% of that in sham rats, respectively (Figure 1B), although intimal thickness remained unchanged. In sham rats, medial thickness and adventitial area were unchanged during the observation period.

Vasa Vasorum Formation
In sham rats, small numbers of AVV were observed in thin adventitia, and BrdU incorporation was not detected in ECs of AVV during the observation period (Figure 2A). In AC rats, BrdU+ ECs were found in the adventitia after day 3. At day 7, the peak increase in BrdU+ ECs was observed, and a large part of AVV with nucleated ECs showed BrdU+ ECs (Figure 2B). At day 28, BrdU+ ECs remained modestly increased. We found many AVV of various sizes in the aortic adventitia of AC rats, and AVV count reached 219% of that in sham rats at day 28 (Figure 2B). In AC rats, most BrdU+ ECs existed in the AVV, whereas isolated BrdU+ ECs were very occasionally found in the adventitial fibrous tissue. Neither vWF+ cell nor vasalike structure was found in the media of AC and sham rats over the course of the study. The aortic luminal ECs did not show BrdU+ incorporation in either group during the observation period.

Adventitial fibroblasts and AVV SMCs also increased in the hypertensive aorta (Table). BrdU+ fibroblasts were scarcely found in the adventitia of intact aorta. At day 7, a robust increase in BrdU+ fibroblasts was noted in the adventitia of AC rats, and fibroblasts accounted for ~90% of BrdU+ adventitial cells. BrdU+ adventitial fibroblasts were evenly distributed and dispersed among the fibrous tissue of the adventitia. Adventitial BrdU+ fibroblast remained modestly increased at day 28. BrdU+ AVV SMCs transiently increased in the hypertensive aorta, although the count of BrdU+ AVV SMCs was relatively smaller than BrdU+ fibroblasts or ECs. At days 7 and 28, single or a few SMCs of AVV showed BrdU incorporation, and in most cases, BrdU+ ECs were observed in the AVV with BrdU+ SMC.

Expression of VEGF Signaling Axis Proteins
Immunoblotting revealed that VEGF and HIF-1α were not detected in the aorta at day 0 (before AC operation; Figure 3A).
At day 7, AC rats clearly showed 2 immunoreactive bands for VEGF, which indicate the 165- and 189-amino-acid splicing variants of VEGF. VEGF expression decreased to lower levels at day 28. Also, HIF-1α/H9251 expression was evident at day 7, returning to insignificant levels by day 28. Sham rats showed no detectable VEGF and HIF-1α/H9251 expression over the course of the study (data not shown).

Immunohistostaining was performed to investigate the temporal and spatial changes in VEGF and HIF-1α (Figure 3B). VEGF was not detected in the aorta at day 0. VEGF expression was first noted in the outer layers of medial SMCs at day 3 and became evident in the outer to middle layers of medial SMCs at day 7. VEGF expression in the thickened media returned to immunohistochemically insignificant levels by day 28. Sham rats showed no detectable VEGF and HIF-1α expression over the course of the study (data not shown).

Immunohistostaining was performed to investigate the temporal and spatial changes in VEGF and HIF-1α (Figure 3B). VEGF was not detected in the aorta at day 0. VEGF expression was first noted in the outer layers of medial SMCs at day 3 and became evident in the outer to middle layers of medial SMCs at day 7. VEGF expression in the thickened media returned to immunohistochemically insignificant levels by day 28. HIF-1α was not found in the aorta at day 0. In AC rats, HIF-1α expression was first observed in the outer layers of medial SMCs at day 3 and extended to the middle layers of medial SMCs at day 7, returning to insignificant levels by day 28.

### Adventitial Proliferating Cells in Hypertensive Aorta

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblasts</td>
<td>11.8±10.7</td>
<td>9270.8±1782.5*</td>
<td>7266.8±1654.3*</td>
</tr>
<tr>
<td>ECs</td>
<td>6.3±8.8</td>
<td>992.3±105.4*</td>
<td>545.0±59.1†</td>
</tr>
<tr>
<td>SMCs in vasa vasorum</td>
<td>1.8±2.4</td>
<td>76.0±18.9*</td>
<td>49.3±19.7†</td>
</tr>
</tbody>
</table>

The number of BrdU* cells is expressed as count per section. Values are mean±SD (n=5).

*P<0.05, †P<0.05 vs days 0 and 7, respectively.

In sham rats, immunoreactive Ets-1 was not found in adventitial ECs. AC induced Ets-1 expression in ECs of AVV (Figure 4a and 4c). Moreover, an immunohistological study of serial tissue sections revealed that BrdU incorporation was observed in most of the Ets-1* ECs of AVV (Figure 4b and 4d). In contrast, there were no apparent changes in the expression of Ets-1 in the aortic luminal ECs in AC and sham rats (data not shown).

### Discussion

Medial thickening and adventitial thickening are well-known characteristics of hypertensive vascular remodeling. However,
Little concern was paid to changes in AVV during hypertensive remodeling. The present study clearly demonstrated that hypertension induces EC proliferation in the adventitia and the resultant AVV formation in the rat aorta. We have also found for the first time that during hypertensive remodeling, concomitant expression of HIF-1α and VEGF was transiently induced in the outer to middle layers of medial SMCs. Furthermore, Ets-1 expression was induced in the proliferative ECs of AVV. Our findings provide insight into AVV formation during hypertensive vascular remodeling.

**VEGF Induction in Hypertensive Aorta**

SMCs have been shown to express and secrete VEGF, but the role of VEGF production by SMCs is currently not well understood. Recent studies suggested that VEGF, which is produced by intimal SMCs, may play a paracrine role in the maintenance and repair of the integrity of the luminal EC lining or in the abnormal proliferation of the intimal/medial vasa vasorum that takes place in atherosclerosis. However, this was not the case in hypertensive remodeling, because VEGF expression was distributed in the outer to middle layers of medial SMCs but not in the intima in our model (Figure 3). It is noteworthy that induction of Ets-1 was accompanied by BrdU incorporation in ECs of AVV but not in the aortic luminal ECs in the hypertensive aorta (Figure 4). VEGF is known to stimulate Ets-1 induction, leading to the expression of genes mediating EC proliferation and their conversion to the angiogenic phenotype. Taken together, it is suggested that VEGF, which is produced in the media, activates ECs of AVV in a paracrine manner and then induces EC proliferation of AVV and new AVV formation in hypertensive aorta. At day 28, weak but consistent VEGF expression was observed on the basis of immunoblotting analysis (Figure 3), which is more sensitive and specific for detecting the protein of interest than immunohistochemical analysis. The VEGF expression is consistent with the presence of BrdU+ ECs at day 28 (Figure 2A).

**HIF-1α Induction in Hypertensive Aorta**

Another new, important finding of the present study was that HIF-1α was transiently expressed in the medial SMCs of the hypertensive aorta with a peak at day 7, concomitant with VEGF (Figure 3). It is noteworthy that HIF-1α and VEGF expression was evident in the outer to middle layers of medial SMCs (Figure 3), which are known to be vulnerable to hypoxia. It has been shown that HIF-1α protein levels increase markedly as cellular oxygen concentration is reduced and decay rapidly when cells are returned to a nonhypoxic environment. The role of HIF-1α in activation of VEGF gene transcription is established in hypoxic cells. Therefore, it is likely from our results that tissue hypoxia secondary to hypertension induces HIF-1α expression, which results in VEGF induction, and that the increase in AVV is a compensatory mechanism for medial hypoxia during hypertensive remodeling. The medial HIF-1α and VEGF expression declined to lower levels at day 28, although further medial thickening was observed (Figures 1 and 3). These findings may suggest that medial hypoxia was compensated for at day 28 by oxygen supply through the increased AVV. This finding seems consistent with an earlier observation that blood flow delivered via vasa vasorum to the thoracic aorta was not different between normotensive and chronically hypertensive dogs in the steady state.

Immunoreactivity for HIF-1α was found in the nuclei of medial SMCs of hypertensive aorta, and cytoplasm was also labeled with HIF-1α in some SMCs with HIF-1α+ nuclei (Figure 3B). The peptide competition test using blocking peptide for the antibody (sc7811p, Santa Cruz Biotech) showed specific inhibition of both nuclear and cytoplasmic stain (data not shown), indicating that the immunoreactivity not only in the nucleus but also in the cytoplasm was specific for HIF-1α. The significance of HIF-1α expression in the cytoplasm of SMCs in hypertensive aorta is currently unknown. Although HIF-1α is a nuclear protein, HIF-1α is synthesized in the ribosomes in the cytoplasm and translocated into the nuclei on the activation. A possible explanation may be that both the nucleus and cytoplasm were labeled with HIF-1α in the present study.

**Possible Mechanisms of VEGF Induction**

Concomitant expression of HIF-1α and VEGF suggests that the HIF-1α-dependent pathway plays a role in VEGF induction in the hypertensive aorta. It is plausible that medial thickening is a major cause of expression of these tissue hypoxia-sensitive factors in hypertensive aorta because (1) the oxygen tension of the arterial wall is inversely related to vessel wall thickness and (2) as medial hypertrophy progresses, the oxygen requirement of the thickened media increases and exceeds the normal oxygen supply that is derived via diffusion from blood in the central lumen. However, the present study revealed that not only HIF-1α and VEGF expression of medial SMCs but also EC proliferation of AVV occurred before medial thickening significantly developed. These observations imply that factors other than medial thickening may contribute to medial hypoxia and AVV formation. First, blood flow through AVV may be restricted in hypertensive vessels during acute increase in arterial pressure. It has been shown that blood flow to the media is reduced in the thoracic aorta of acutely hypertensive dogs with suprarenal aortic clamping, suggesting that deformity or stretch of the aortic wall might compress the AVV and create impaired medial oxygen supply. Second, the oxygen requirement of medial SMCs may be increased in response to hypertension because hypertension induces neurohumoral activation and local generation of various cytokines, growth factors, and vasoactive substances, all of which activate SMCs and subsequently increase cellular oxygen consumption. Moreover, a very recent study has shown that growth factors induce HIF-1α expression through the phosphatidylinositol 3-kinase/Akt pathway in some cell types other than SMCs. In cultured SMCs, VEGF expression is upregulated by cytokines and growth factors. Thus, growth factor– or cytokine-induced HIF-1α activation may be involved in VEGF expression of vessels during hypertensive remodeling. This possibility should be tested in SMCs in future studies.

Additionally, it is possible that the HIF-1α-independent pathway is involved in the mechanisms of VEGF induction in hypertensive aorta. It is conceivable that the aortic wall stretch induced by hypertension may enhance VEGF expression in medial SMCs through an HIF-1α-independent pathway because an ex vivo study has shown that mechanical
stretch directly induces VEGF expression in the rat carotid artery via a protein kinase C–mediated mechanism.\textsuperscript{29} Moreover, hypoxia-induced oxidative stress\textsuperscript{35} is another possible HIF-1α–independent mechanism of VEGF induction, because oxidative stress has been implicated in VEGF expression in the rat carotid artery after vascular injury.\textsuperscript{30} Further investigations are needed to clarify the mechanisms regulating VEGF expression in hypertensive arteries.

\textbf{Study Limitations}

First, earlier studies established that blood vessels $<500$ μm (the "critical depth") do not contain demonstrable intramural medial/intimal vasa vasorum.\textsuperscript{31} In the intact and AC rats in our study, medial thickening did not exceed the critical depth, and vasa vasorum formation was not detected in the media and intima at least by day 28. Thus, aortic remodeling of AC rats is considered a model of hypertensive arteriosclerosis of arteries that have no medial/intimal vasa. However, this rat model does not seem adequate to investigate the role of medial and intimal vasa vasorum in hypertensive or atherosclerotic remodeling of arteries with a medial thickness exceeding the critical depth, which is typically observed in the aorta and in the coronary or femoral artery of large animals, including humans. To address this issue, hypertension models should be established in larger animals.

Second, we did not measure blood flow through vasa vasorum within the aortic wall. Thus, it remains to be clarified whether blood flow within the aortic wall correlates with AVV count. Third, the present study demonstrated that adventitial fibroblasts and AVV SMCs also increased in the hypertensive aorta. Because VEGF has no direct effect on fibroblast and SMC proliferation, the mechanisms of the proliferation of these cells and the interaction among EC, SMC, and fibroblast proliferation remain to be elucidated in future study.

In conclusion, hypertension increased AVV count, concomitant with medial and adventitial thickening, in the rat aorta. A role of the HIF-1α/VEGF/Ets-1 pathway is suggested in the adventitial EC proliferation and resultant AVV formation. These observations imply that AVV play a beneficial role in the maintenance of adequate oxygen supply for the aortic wall during vascular remodeling in response to hypertension.

\textbf{Acknowledgments}

This study was supported in part by a Science Frontier Research Promotion Centers grant from the Ministry of Education, Science, Sports, and Culture of Japan and by Japan Heart Foundation and Pfizer Pharmaceuticals grant for research on coronary artery disease (Dr Kai). We thank Kaoru Moriyama and Yayoi Yoshida for technical assistance.

\textbf{References}

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Hypertension. 2002;39:46-50
doi: 10.1161/hy1201.097200

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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