Abstract—Angiotensin II (Ang II) upregulates vascular endothelial growth factor (VEGF) and activates vascular inflammation. However, the decisive role of VEGF in Ang II–induced vascular inflammation and remodeling has not been addressed. Ang II infusion to wild-type mice increased local expression of VEGF and its receptors in cells of aortic wall and plasma VEGF, and caused aortic inflammation (monocyte infiltration) and remodeling (wall thickening and fibrosis). Hypoxia-inducible factor-1α colocalized with VEGF-positive cell types. Blockade of VEGF by the soluble VEGF receptor 1 (sFlt-1) gene transfer attenuated the Ang II–induced inflammation and remodeling. The sFlt-1 gene transfer also inhibited the increased expression of VEGF and inflammatory factors such as monocyte chemoattractant protein-1. In contrast, sFlt-1 gene transfer did not affect Ang II–induced arterial hypertension and cardiac hypertrophy. VEGF is an essential mediator in Ang II–induced vascular inflammation and structural changes through its proinflammatory actions. (Hypertension. 2004;44:264-270.)

Key Words: growth substances ▪ arteriosclerosis ▪ remodeling ▪ angiotensin II ▪ endothelial growth factors

Activation of the renin-angiotensin system as a result of impaired endothelial function plays an important role in the initiation and progression of arteriosclerosis/atherosclerosis through multiple mechanisms.1–3 We have shown that chronic inhibition of nitric oxide synthesis upregulates angiotensin II (Ang II) production and expression of Ang II type-1 (AT₁) receptor, resulting in vascular inflammation and arteriosclerosis in a rat model.1,4–7 Ang II augments production of inflammatory cytokines and chemokines by arterial wall cells and monocytes.8–10 Furthermore, emerging evidence suggests that Ang II is implicated in the process of angiogenesis.11 Ang II is shown to upregulate vascular endothelial growth factor (VEGF) and promote tumor-associated, VEGF-induced, ischemia-induced angiogenesis in vitro and in vivo.12–14 There is no report, however, that addressed the role of VEGF in Ang II–induced vascular inflammation and structural changes under in vivo conditions.

VEGF is one of the most potent angiogenic factors known to date and is thought to function as an endogenous regulator of endothelial integrity.15–18 Previous animals studies have reported that local delivery of VEGF after endothelial injury promotes endothelial regeneration, accelerates the recovery of endothelium-dependent relaxation, and reduces neointimal formation.18 However, there is still a considerable debate over the vasculoprotective versus pro-inflammatory/arteriosclerotic effects of VEGF.18 There is emerging evidence that VEGF induces migration and activation of monocytes through induction of adhesion molecules or chemokines such as monocyte chemoattractant protein-1 (MCP-1).19,20 and that VEGF enhances neointimal formation by stimulating intraplaque angiogenesis21–23 or by increasing inflammation.24 Therefore, vasculoprotective versus proinflammatory/arteriosclerotic actions of VEGF remains to be inconclusive.

Accordingly, we aimed to determine the decisive role of VEGF in Ang II–induced vascular remodeling (medial thickening and hypertrophy) in vivo. To determine the role of VEGF in vivo, we used a soluble form of the VEGF receptor-1 (sFlt-1) that blocks VEGF activity by directly sequestering VEGF and by functioning as a dominant-negative inhibitor against VEGF.25,26 We and other investigators have demonstrated that intramuscular transfection of sFlt-1 gene effectively blocks VEGF, and thus quenches activity of VEGF in vivo.27,28 We report here that sFlt-1 gene transfer attenuated Ang II–induced vascular inflammation and remodeling in mice. The present study seems to be the first in vivo evidence for an essential role of VEGF in the pathogenesis of Ang II infusion-induced vascular inflammation and remodeling.

Methods

Expression Vector

The 3.3-kb mouse sFlt-1 gene was obtained from a mouse lung cDNA library29 and cloned into the BamH1(5’) and NotI(3’) sites of the eukaryotic expression vector plasmid cDNA3 (Invitrogen).
Experimental Animals

The study protocol was reviewed and approved by the Committee of the Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences. A part of this study was performed at the Kyushu University Station for Collaborative Research and the Morphology Core, Kyushu University School of Medicine Sciences.

Treatments

Male C57BL/6J wild-type mice were purchased from Jackson Laboratory (Bar Harbor, Me) and fed with commercial standard chow. Mice at 8 to 10 weeks old were randomly divided into 5 groups: (1) the untreated control group; (2) ones receiving Ang II infusion; (3) Ang II infusion plus sFlt-1 gene plasmid transfer; (4) Ang II infusion plus empty plasmid cDNA3 transfer; or (5) Ang II infusion and Ang II AT1 receptor blocker (olmesartan at 3.5 mg/kg per day) was implanted in the peritoneal cavity under anesthesia with ketamine (80 mg/kg IP) and xylazine (10 mg/kg IP). Treatment with olmesartan started 3 days before Ang II administration was begun. For gene transfer, either empty plasmid or sFlt-1 plasmid (150 μg/100 μL phosphate-buffered saline per mouse) was injected into both sides of femoral muscles using a 27-gauge needle 1 day before commitment of Ang II infusion, as we previously described.27,29,30

In all experiments, mice were euthanized at the indicated time points of treatments for analysis. Venous blood was collected immediately before the mice were euthanized. The aorta and hearts were isolated and either fixed in 10% buffered formalin or snap-frozen. Systolic blood pressure was measured by the tail-cuff method.

Immunohistochemistry, Histopathology, and Morphometry

Immunohistochemistry and histopathology were performed as described previously.6,29 Some of formalin-fixed and paraﬃn-embedded cross-sections of abdominal aorta were routinely stained with hematoxylin-eosin or Masson-trichrome. The other sections were subjected to immunostaining assay using antibodies against mouse VEGF and its receptors, Flt-1 and Flk-1 (Santa Cruz Biotech), macrophages (Mac-3; Serotec Inc, Raleigh, NC), proliferating cell nuclear antigen (DAKO, Denmark), α-smooth muscle cell actin (α-SMA; Boehringer Mannheim, Germany), hypoxia-inducible factor-1α (HIF-1α), CD31 (Santa Cruz Biotech), and von Willebrand factor (Sigma Chemical).

Fluorescein FITC-conjugated or rhodamine-conjugated secondary antibodies (Santa Cruz Biotech) were used for double-staining for localization of cell types expressing VEGF and its 2 receptors, or for coexpression of VEGF and HIF-1α. The degree of arteriosclerosis (the medial thickness and perivascular fibrosis) and left ventricle (LV) hypertrophy (LV-to-body weight ratios) on day 28 were measured as described previously.4,5,7

TaqMan Real-Time Reverse Transcription–Polymerase Chain Reaction

Transcripts of 1 μg total RNA from thoracic and abdominal aorta were reverse-transcribed and the resultant cDNA was amplified by TaqMan real-time reverse transcrip–polymerase chain reaction as previously described50 for the following genes: VEGF, Flt-1, Flk-1, HIF-1α, B-type natriuretic peptide, MCP-1, CC2R (MCP-1 receptor), interleukin-1 (IL-1), IL-6, transforming growth factor β-1 (TGF-β1), intercellular adhesion molecule-1, and vascular cell adhesion molecule-1. The sequences of sense primers, antisense primers, and the relevant probes were recorded (online Table I available at http://www.hypertensionaha.org). The probe and primers of GAPDH were obtained from Applied Biosystems.

Plasma VEGF and sFlt-1 Measurements

The commercially available enzyme-linked immunosorbent assay kits (Biosource International, Camarillo, Calif) were used to measure

Figure 1. Local and systemic expression of VEGF during Ang II infusion. A, Time courses of Ang II-induced morphological immunohistochemical changes. Cross-sections of abdominal aorta stained with Masson-trichrome (M-T) or with antibodies against VEGF, its 2 receptors Flt-1 and Flk-1, and HIF-1α (positive staining are yellow–brown) are shown. The micrographs at the right of each immunohistochemical staining show negative staining with nonimmune-IgG. *Luminal sides of aorta. The black lines indicate external elastic lamina (EEL). Scale bar=50 μm. B, Time courses of mRNA expression of VEGF and Hif-1α in aorta (Ao) and left ventricle (LV). The mRNA value of VEGF or Hif-1α was normalized by GAPDH mRNA in each sample (n=6 to 8). **P<0.05, ***P<0.01 vs the untreated control. C, Time course of plasma VEGF level before and after Ang II infusion (n=6 to 8). *P<0.05, **P<0.01 vs the untreated control; †P<0.01 vs Ang II group.
mouse plasma VEGF and soluble Flt-1 according to the manufacturer’s instructions.

Statistical Analysis
Data are expressed as the mean±SE. Statistical analysis of differences was compared by analysis of variance. Post hoc analyses were performed using Bonferroni correction for multiple comparison tests. \( P<0.05 \) was considered to be statistically significant.

Results
Expressions of VEGF, Flt-1, Flk-1, and HIF-1α
Compared with no staining in aortic sections from control mice, intense immunohistochemical staining of VEGF, Flt-1, and Flk-1 were seen in aortic sections from mice with Ang II infusion mainly in inflammatory lesions (mononuclear cell infiltration) of the adventitia at an early (days 3 and 7) phase of Ang II infusion (Figure 1A). On day 28, cells in the media also became positive with VEGF and its 2 receptors (Figure 1A). Gene expression of VEGF markedly increased in the aorta and LV. It peaked on day 7 and then spontaneously declined on day 28 (Figure 1B). Because HIF-1α is a transcriptional factor for the control of VEGF expression, \( \text{HIF-1α} \) immunostaining and mRNA levels of HIF-1α were then examined. HIF-1α expression showed similar temporal and special changes as those of VEGF (Figure 1A and 1B).

Figure 2. Colocalization of cell types with VEGF and its receptors through immunofluorescent double-staining. A, Some Mac-3–positive macrophages recruited into the adventitia express VEGF or its receptor Flt-1 (VEGF-R1) on day 7. B, VEGF and HIF-1α colocalize in same cell types on day 7. C, \( \alpha \)-SM actin–positive cells in medial smooth muscle cells, and adventitial myofibroblast-like cells express VEGF or its receptors on day 28. The white lines indicate EEL or internal elastic lamina (IEL) of aorta. Scale bar=50 \( \mu \text{m} \).
Keeping with rapid upregulation of VEGF in vascular tissues, serial measurements of plasma VEGF showed rapid and persistent increase in the Ang II group (Figure 1C).

To localize VEGF and related signaling, immunofluorescent double staining was performed (Figure 2). On days 3 and 7, Mac-3-positive monocytes recruited to the adventitia and outer layer of the media expressed VEGF and Flt-1 (Figure 2A), but did not express Flk-1 (data not shown). HIF-1α was colocalized in the cell types expressing VEGF (Figure 2B). On day 28, most α-SMA-positive smooth muscle cells in the media expressed VEGF, and some α-SMA-positive cells in the media expressed Flt-1 and Flk-1 (Figure 2C). Some α-SMA-positive myofibroblastic cells in the adventitia expressed VEGF, Flt-1, and Flk-1 (Figure 2C).

No apparent angiogenesis, as detected by von Willebrand factor or CD31 staining, was detected in the aortic wall of the control, Ang II, or Ang II+empty plasmid groups (data not shown). Furthermore, the endothelial layer of the aorta was preserved in the 3 groups.

**Effects of sFlt-1 on Vascular Inflammation and Remodeling**

Mac-3-positive monocytes and proliferating cell nuclear antigen-positive proliferating cells were used as the markers of inflammatory and proliferative changes. Infiltration of monocytes and appearance of proliferating cells was markedly increased in the aorta of mice receiving Ang II, particularly in the adventitia on days 3 and 7, which declined spontaneously on day 28. These Ang II–induced inflammatory and proliferative changes in the aorta on day 7 were markedly attenuated in Ang II+sFlt-1 group, but not in the Ang II+empty plasmid group (Figure 3).

Compared with control mice, vascular remodeling (medial wall thickening and perivascular fibrosis) developed in the aorta and coronary arteries from mice received Ang II for 28 days, which was attenuated by sFlt-1 gene transfer but not by empty plasmid transfer (Figure 4A and 4B).

To gain mechanistic insight, mRNA levels of a variety of inflammatory cytokines, chemokines, and chemokine receptors were examined by real-time polymerase chain reaction on day 7 (Figure 5). The sFlt-1 transfection did not affect the increased gene expression of RANTES, MIP-1α, or MIP-2, but prevented or attenuated the increased gene expressions of VEGF, Flt-1 Flk-1, MCP-1, CCR2, IL-1β, IL-6, TGF-β1, vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and HIF-1α (Figure 5). The sFlt-1 transfection...
also blunted Ang II–induced increases in plasma VEGF (Figure 1C). Immunohistochemical study revealed that immunoreactive MCP-1, TGF-β1, VEGF, Flt-1, and Flk-1 were increased in Ang II group on day 7 (Figure 6). In contrast, such increased immunostaining was attenuated in aortic sections from the Ang II+sFlt-1 group.

Plasma sFlt-1 Concentration
To assess transfection efficacy, plasma sFlt-1 concentration was measured. In control mice, plasma sFlt-1 levels increased on days 3, 7, and 14 (Table 1), indicating that sFlt-1 was released to circulation from the transfected muscle. Similar increase in plasma sFlt-1 levels was noted in mice infused with Ang II.

Systolic Blood Pressure and LV Hypertrophy
Systolic blood pressure was significantly increased in mice receiving Ang II compared with control. There were no significant differences in systolic blood pressure between Ang II and Ang II+sFlt-1 groups (Table 2). To assess the degrees of LV hypertrophy, relative LV weight and B-type natriuretic peptide mRNA levels were determined on day 28 (Table 2). There were no significant differences in Ang II–induced LV hypertrophy or in the increase in B-type natriuretic peptide mRNA levels between Ang II and Ang II+sFlt-1 groups.

Effects of AT1 Receptor Blocker on Vascular Inflammation and Remodeling
Treatment with AT1 receptor blocker prevented or markedly attenuated Ang II–induced arterial hypertension, LV hypertrophy (Table 2), and increased immunostaining and gene expression of VEGF, aortic wall inflammation, and arteriosclerotic changes (data not shown). These data suggest that the Ang II–induced increases in VEGF expression and activity were mediated by Ang II AT1 receptor stimulation.

**Table 1. Plasma Concentrations of sFlt-1**

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>Baseline</th>
<th>3</th>
<th>7</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>467±37</td>
<td>1037±132*</td>
<td>927±215*</td>
<td>649±83*</td>
</tr>
<tr>
<td>Ang II</td>
<td>Not measured</td>
<td>370±23</td>
<td>393±35</td>
<td>383±29</td>
</tr>
<tr>
<td>Ang II+sFlt-1</td>
<td>Not measured</td>
<td>1006±22*</td>
<td>1297±24*</td>
<td>730±26*</td>
</tr>
</tbody>
</table>

*Mean±SEM (n=6).

*P<0.01 vs. baseline in untreated control mice.
TABLE 2. Systolic Blood Pressure, Relative Left Ventricle Weight, and Brain Natriuretic Peptide Gene Expression

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>Day 7</th>
<th>Day 28</th>
<th>LV/BW Ratio (Day 28)</th>
<th>BNP/GAPDH Ratio (Day 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>119±3</td>
<td>120±5</td>
<td>4.36±0.12</td>
<td>1.04±0.06</td>
</tr>
<tr>
<td>Ang II</td>
<td>176±7*</td>
<td>185±8*</td>
<td>5.12±0.20*</td>
<td>1.91±0.07*</td>
</tr>
<tr>
<td>Ang II+ARB</td>
<td>113±6†</td>
<td>117±9†</td>
<td>4.45±0.18†</td>
<td>0.97±0.08†</td>
</tr>
<tr>
<td>Ang II+sFlt-1</td>
<td>173±9*</td>
<td>180±12*</td>
<td>5.06±0.11*</td>
<td>2.03±0.15†</td>
</tr>
</tbody>
</table>

SBP indicates systolic blood pressure; LV, left ventricle; BW, body weight; BNP, B-type natriuretic peptide. Mean±SEM (n=6 to 8).
*P<0.01 vs. control; †P<0.01 vs. Ang II.

Discussion

Ample evidence suggests that VEGF-mediated signals are essential in Ang II–induced angiogenesis in vivo and endothelial migration/proliferation in vitro.12–14 The functional importance of VEGF in the mechanism of Ang II–induced vascular inflammation and structural changes, however, has not been addressed. We report here that sFlt-1 gene transfer attenuated the Ang II–induced vascular inflammation and structural changes (medial wall thickening and fibrosis) in mice. Therefore, the present study provides the first in vivo evidence to our knowledge for an essential role of VEGF in the pathogenesis of Ang II infusion-induced inflammation and remodeling.

We examined time-related changes in cell types expressing VEGF and its receptors during Ang II infusion. VEGF was predominately expressed in the lesional monocytes and proliferative myofibroblast, mainly in the adventitial layer at early stages and in smooth muscle cells in the media. This local VEGF expression was associated with rapid and persistent increase in plasma VEGF level. In addition, Flt-1 was increased in lesional monocytes and medial smooth muscle cells at early stages and in medial smooth muscle cells at later stages. No increase in Flk-1 expression was detected in monocytes or myofibroblasts, whereas increased Flk-1 expression was noted in medial smooth muscle cells only at later stages. Our present data show that Ang II–mediated expressions of VEGF and its receptors have a biological effect in inducing vascular inflammation (monocyte infiltration) and proliferation, as well as in causing vascular structural changes. Interestingly, sFlt-1 gene transfer reduced increased local and systemic expression of VEGF, suggesting that sFlt-1 transfection might inhibit VEGF activity at least by trapping VEGF. Because sFlt-1 functions as a nonselective inhibitor of Flt-1 and Flk-1, further studies are needed to elucidate relative role of Flt-1 and Flk-1 in the pathogenesis of Ang II–induced vascular pathobiology.

There are several reports demonstrating that VEGF is a proinflammatory factor.20,32 In the present study, we extended those observations by showing that sFlt-1 gene transfer attenuated Ang II–induced increase in inflammatory factors in vivo. Regarding the mechanism of VEGF-mediated vascular inflammation, Yamada et al15 showed that MCP-1 is essential in VEGF-induced angiogenesis and inflammation. Bush et al24 showed that Ang II–induced vascular inflammation and arteriosclerosis was blunted in mice deficient of MCP-1 receptor. MCP-1 has been shown to be the key chemokine in mediating vascular monocyte-mediated inflammation leading to vascular disease.35,36 Taken together, it is likely that sFlt-1 gene transfer blocked Ang II–induced vascular structural changes mainly by suppressing inflammation (monocyte recruitment and activation) and subsequent production of growth factors. For example, VEGF-mediated overexpression of TGF-β1 might contribute to Ang II–induced vascular fibrosis. Another interpretation alternative to this conclusion is that increased VEGF and its receptors acted directly on smooth muscle cells, resulting in structural changes such as medial thickening. Several studies have reported that VEGF has direct actions on proliferation/migration of smooth muscle cells,37,38 which may not be mediated by inflammation (monocyte recruitment). It is possible therefore that some of the mechanism by which sFlt-1 gene transfer inhibited vascular structural changes might not be caused by inflammation.

Regarding the mechanism of Ang II–induced expression of VEGF, we examined HIF-1α expression because HIF-1α plays a major role in the control of VEGF expression. Richard et al31 reported that Ang II induces VEGF production through HIF-1α in vascular smooth muscle cells in vitro. In the present study, we showed that Ang II infusion increased local HIF-1α expression in vascular wall cells that colocalized in VEGF-expressing cells types, suggesting that increased transcription of HIF-1α is involved in Ang II–induced expression of VEGF.

It is noteworthy that sFlt-1 gene transfer did not affect Ang II–induced arterial hypertension or indices of left ventricular hypertrophy. Arterial blood pressure was, however, measured by the tail-cuff method, a method that cannot provide reliable measure of the pressure changes associated with Ang II infusion. It is reported that arterial hypertension contributes to Ang II–induced vascular remodeling.39 Furthermore, the dose of Ang II used in the present study was high, which is above the range of physiological condition. Nevertheless, our present observation suggests that VEGF may not be involved in the mechanism of Ang II–induced hypertension or cardiac hypertrophy.

Perspectives

VEGF is likely to be an essential mediator in Ang II–induced vascular inflammation and remodeling but is not involved in Ang II–induced cardiac hypertrophy. Our present data support the notion that VEGF acts as a proinflammatory and proatherosclerotic factor in Ang II–induced hypertension.

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Essential Role of Vascular Endothelial Growth Factor in Angiotensin II–Induced Vascular Inflammation and Remodeling
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