Negative Regulation of VEGF-Induced Vascular Leakage by Blockade of Angiotensin II Type 1 Receptor

Hideto Sano, Kohei Hosokawa, Hiroyasu Kidoya, Nobuyuki Takakura

Objective—Permeability of blood vessels is essential for tissue homeostasis. However, disorganized hyperpermeability leads to progression of diseases. Vascular endothelial growth factor-A (VEGF) is a key regulator for leakiness of blood vessels and it has been reported that VEGF-mediated hyperpermeability was suppressed by angiopoietin-1 (Ang1). We found that Angiopoietin-converting enzyme (ACE) was downregulated in endothelial cells by Ang1. ACE converts angiogenisin I to angiogenisin II (AngII). Here, we studied the relationship between VEGF and AngII relative to vascular permeability.

Methods and Results—We showed that VEGF-mediated vascular hyperpermeability was suppressed in mice given AngII type 1 receptor (AT1R) blocker (ARB); the effect was also seen in AT1R-deficient mice. In this system, we found that ARB inhibited VEGF-induced gap formation. Furthermore, we ascertained that angioedema induced by overexpression of VEGF decreased noticeably in ARB-treated ischemic mice.

Conclusions—Because ARB suppressed VEGF-induced vascular hyperpermeability, we propose that ARB may be used to minimize the risk of edema in therapeutic angiogenesis using VEGF. (Arterioscler Thromb Vasc Biol. 2006;26:2673-2680.)

Key Words: VEGF ● Tie2 ● angiogenitin-converting enzyme ● gene therapy ● VE-cadherin

It has been widely believed that vascular endothelial growth factor-A (VEGF) can rapidly induce hyperpermeability in the adult and that VEGF-induced vascular leakage through VEGF receptor, VEGFR2/Fk-1, may lead to edema and swelling and contribute to many disease processes including brain tumors, diabetic retinopathy, ischemic strokes, sepsis, and also inflammatory conditions such as rheumatoid arthritis and asthma. It has been reported that the vascular leakage induced by VEGF could be blocked by acute administration of angiopoietin-1 (Ang1). Ang1 and VEGF are endothelial cell (EC)-specific growth factors. Ang1 induces angiogenic signals through the Tie2 receptor tyrosine kinase, which is expressed on ECs and hematopoietic stem cells. An Ang1 relative, termed angiopoietin-2 (Ang2), was shown to be a naturally occurring antagonist for Ang1 and Tie2. Ang1 and Ang2 are essential for normal vascular development in the mouse. VEGF also has been shown to be required for blood vessel formation during embryogenesis and to augment postnatal angiogenesis. Despite the fact that VEGF was initially called vascular permeability factor, there has been less focus on its permeability actions and more efforts have been devoted to its clinical application for ischemia and cancer. Actually, direct comparison of transgenic mice overexpressing VEGF in the skin revealed that blood vessels induced by VEGF were leaky, whereas those induced by Ang1 were not leaky. Furthermore, blood vessels in Ang1-overexpressing mice were resistant to leakiness caused by inflammatory stimulation and VEGF. In contrast to Ang1, transgenic overexpression of Ang2 on ECs disrupts blood vessel formation by disrupting the interaction of ECs and mural cells (MCs) in the mouse embryo; this is also observed in Tie2- or Ang1-null mice. In the adult human and the rat, Ang2 is expressed only at sites of vascular remodeling such as hypoxic areas. In the sprouting angiogenesis induced by hypoxia, Ang2 expression on ECs has been suggested to disrupt the interaction of ECs and MCs and to initiate sprouting of ECs from preexisting vessels. Moreover, it was reported that a mutation of Tie2 in humans causes venous malformations, which typically is a result of an imbalance of ECs and MCs. Inhibition of MC recruitment by anti-PDGF β receptor neutralizing antibody resulted in pericyte loss; however, administration of Ang1 in this situation reduced the vascular leakage. These findings suggest that the angiopoietin/Tie2 system has a central role in endothelial–mural cell interaction and regulates vascular permeability as well as maturation and stabilization of vascular structures. Although Ang1 protein administration has potential therapeutic applications in en-
hancing EC survival and inhibiting vascular leakage, the clinical use of this factor has several potential problems. Large-scale production of recombinant Ang1 protein is hindered by the loss of its activity by aggregation and insolubility of the protein. Moreover, the properties of the protein frequently vary after purification. These difficulties are attributable to its unique structural characteristics. Although it has been reported that the COMP-Ang1, which is a potent Ang1 variant produced by replacing the N-terminal portion of Ang1 with the short coiled-coil domain of cartilage oligomeric matrix protein, induces angiogenesis, vascular enlargement, and lymphangiogenesis, this factor is still under development for clinical use. Therefore, other candidate molecules that inhibit vascular permeability are sought for use in combination with VEGF in therapeutic angiogenesis.

Moreover, angiotensin II (AII), which is known as a regulator of blood pressure, also has some effects on vascular remodeling including vascular stabilization. Like VEGF, AII increases vascular permeability via the release of prostanoids or direct rearrangement of cytoskeletal proteins. There are two major subtypes of AII receptors, AT (AII Type) 1 and AT2. In mice, AT1 receptor is further subdivided into AT1a and AT1b receptors. Most of the well-known AII functions in the cardiovascular system are mediated through the AT1 receptor, especially through the AT1a receptor subtype in rodents. It was recently reported that AT1a receptor knockout (KO) mice showed reduced systemic blood pressure. In this regard, it is of interest that Sasaki et al showed impairment of hypoxia-induced angiogenesis in AT1a receptor KO mice.

For the clinical management of angiogenesis, the regulatory mechanism of the vascular permeability should be taken into consideration. Because Ang1, VEGF, and AII play central roles in vascular permeability, in the current study, we determined the relationship between Ang1/Tie2, VEGF/VEGFR2, and AII/AT1 receptor systems in vascular permeability in vitro and in vivo. Furthermore, we confirmed the effects of the AT1 receptor antagonists on vascular edema induced by VEGF gene transfer in mice.

### Materials and Methods

#### Animals

AT1a receptor-deficient (AT1a KO) mice (C57BL/6 mice backcrosses) were provided from Tanabe Seiyaku. ICR and C57BL/6 mice were purchased from Japan SLC (Shizuoka). Animals were housed in environmentally controlled rooms of the animal experimentation facility at Kanazawa University. All experiments were carried out under the guidelines of Kanazawa University Committee for animal and rDNA experiments.

#### ACE mRNA Expression

Human microvascular ECs (HMVECs; Kurabo) pretreated with either Ang1 (100 ng/mL) or VEGF (50 ng/mL) for 8 hours were collected and total RNA was isolated using the RNeasy plus kit (Qiagen). Total RNA was reverse transcribed using the reverse transcriptase from ExScript RT reagent Kit (Perfect Real Time) (Takara). ACE mRNA levels of HMVECs were quantified with real-time RT-PCR using fluorescent SYBR green (Platinum SYBR Green qPCR; Invitrogen) and Mx3000P (Stratagene). The sequences of the gene-specific primers for PCR were as follows: 5'-cggagggcagcttgaggaac-3' (sense) and 5'-ggagatggctgagtctactaa-3' (antisense) for ACE and 5'-gaagtggaattctgg-3' (sense) and 5'-gaagaggtcgtggtactttc-3' (antisense) for GAPDH.

#### Assay of ACE Activity

ACE activity was measured using a fluorometric assay as previously described. The bovine aortic ECs (BAECs; Cell Systems) were stimulated with VEGF (50 ng/mL) and/or Ang1 (100 ng/mL). After 72 hours incubation, the cell culture medium were collected and incubated with substrate (Z-Phe-His-Leu) (Bachem Bioscience) for 3 hours at 37°C. The reaction was terminated with NaOH and cleaved His-Leu products were derivatized with O-pthalaldialdehyde (Sigma) and HCl was added. The fluorescence of samples was estimated spectrofluorometrically using Fluoroskan Ascent (Laboratory systems).

#### Plasma Extravasation

To determine the vascular permeability, a Miles assay was performed in mice. Mice (ICR, C57 BL/6 or AT1a KO) were anesthetized and shaved. After 2 to 3 days, mice were anesthetized again and intravenously injected with 150 μL of 1% Evans blue dye. After 15 minutes, intradermal injection of one of the following was performed: 15 μL of VEGF (100 ng/mL), histamine (100 μM/L), AT1 receptor antagonist (50 or 250 μg/mL) (ARB; Candesartan; provided from Takeda Pharmaceuticals), AT2 receptor antagonist (50 or 250 μg/mL) (PD123319; Sigma), or PBS as a negative control. In the inflammatory stimulation study, mustard oil was applied to the dorsal and ventral surface of the ear with a cotton tip. After 1 hour, the skins and ears were photographed, and then they were dissected. The dye was then eluted from the dissected samples with formamide at 56°C, and the optical density was measured by spectrophotometry (Biotrak II, Amersham Biosciences) at 620 nm.

#### Immunohistochemistry for VE-Cadherin and ZO-1

HMVECs were grown to the confluent, serum starved, and stimulated with various factors. The cells were then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were labeled with anti–VE-cadherin antibody or anti–ZO-1 antibody (BD Biosciences) and developed with phycoerythrin conjugated goat anti-rat IgGs (Biosource) for anti–VE-cadherin or with biotin conjugated goat anti-mouse IgGs (DAKO) for anti–ZO-1. Biotin conjugated secondary antibody was visualized with streptavidin-phycoerythrin (BD Biosciences). Nuclei were stained with Hoechst 33258 (Sigma).

#### Intramuscular Naked Plasmid Injection

Plasmid vectors expressing either LacZ (pSh-LacZ) or human VEGF165 DNA (pSh-VEGF) under the influence of the CMV promoter were constructed. The mice were randomly assigned to receive approximately 150 μg (100 μL of saline) of either pSh-LacZ or pSh-VEGF by intramuscular injection at 3 sites on the thigh along the projection of the femoral artery. Injections were performed by direct intramuscular injection into the ischemic or nonischemic hindlimbs. For the verification of gene induction, LacZ expression was confirmed with X-gal staining (data not shown). In the ischemia model, mice underwent surgical cutting at the site of deep femoral artery using bipolar tweezers. Mice also received intravenous injections of saline containing ARB (0.3 μg/mL; dissolved by DMSO) or DMSO every other day. Tissues were embedded in OCT compound and cryosections were cut and stained. Immunohistochemical staining was performed with anti–PECAM-1/CD31 (BD Bioscience) and anti-fibronectin (Sigma) antibodies in the gastrocnemius muscles. Evaluations of PECAM-1/CD31-stained capillary densities were performed by counting blood vessels in 6 random fields of each section.

#### Statistical Analysis

All data were displayed as the mean±SD and were analyzed by repeated-measures two-way ANOVA or Student t test using Statview software (Abacus Concepts). A probability value of less than 0.05 was considered statistically significant.
Results

ACE mRNA Expression and Activity of ECs Were Reduced by Ang1 Stimulation

To investigate the relationship of Ang1/Tie2 system and VEGF/Flk-1 system in the regulation of AII/AT1a receptor system, we first examined whether Ang1 or VEGF affect mRNA expression and activity of ACE, which converts Angiotensin I to AII, in ECs. We found that ACE mRNA decreased on stimulation of Ang1 in HMVECs (Figure 1A). By contrast, mRNA expression of ACE increased in VEGF-stimulated ECs as previously reported (Figure 1A). We further confirmed ACE mRNA expression in BAECs as well as HMVECs (data not shown). Furthermore, ACE activity in BAECs was reduced by Ang1 compared with the basal level (Figure 1B). By contrast, VEGF induced ACE activity in a dose dependent manner. However, VEGF-mediated upregulation of ACE activity was suppressed by simultaneous addition of Ang1. Taken together, these results indicated that Ang1 and VEGF have opposing effects in the regulation of ACE. Moreover, as both VEGF and AII are known to promote vascular permeability, our data suggests that one of the mechanisms by which VEGF induces hyperpermeability is caused by AII upregulation through regulation of ACE expression and activity.

Effects of Blockade of AII Receptors on Vascular Permeability

Because Ang1 suppresses ACE expression and activity, we next studied whether blockade of AII signaling pathway through the AT1 receptor, which plays a major role in hypertensive function, could affect vascular leakage in vivo (Figure 2). There are two possible ways to inhibit the AII/AT1 receptor system; an ACE inhibitor or an ARB. However, for the direct assessment of AT1 receptor function, we used ARB, because ACE inhibitors upregulate bradykinin, which is known to induce vascular permeability. In vivo vascular permeability was determined in mice using the Miles assay. The extravasation of Evans blue induced by VEGF was apparently reduced by ARB in a dose dependent manner (Figure 2A). The leakage of dye decreased significantly from 59.07 ± 12.28 (VEGF alone) to 40.5 ± 6.65 (VEGF plus 250 µg/mL ARB) ng/mg tissue (P < 0.02). In contrast, an antagonist of AT2 receptor (PD 123319), which is another AII receptor subtype, did not alter the vascular permeability induced by VEGF (50.86 ± 10.07 and 43.3 ± 11.26 ng/mg tissue in mice injected with VEGF and VEGF plus 250 µg/mL AT2 receptor antagonist, respectively) (Figure 2B).

Figure 1. Effects of Ang1 and VEGF on ACE mRNA expression and activity in the ECs. A, ACE mRNA expressions in HMVECs were measured by real-time RT-PCR. GAPDH was used as an internal control (means ± SD, n = 3). *P < 0.05 vs control. B, BAECs were stimulated with VEGF (0 to 100 ng/mL) and Ang1 (0 to 100 ng/mL) and ACE activity was determined. The relative ratios of ACE activity are shown (means ± SD, n = 3). *P < 0.05.

Figure 2. Involvement of AT1 receptor in VEGF-induced vascular permeability in the Miles assay. A, Representative photographs of the vascular leakage induced with VEGF in the presence or absence of ARB in ICR mice skin dermis (upper panel). 15 µL PBS with or without VEGF (100 ng/mL) or ARB (50, 250 µg/mL) was intradermally injected. Quantitative analysis of extravasation of Evans blue dye in the skin (mean ± SD, n = 5) (bottom panel). *P < 0.05. B, Representative photographs of the vascular leakage induced with VEGF in the presence or absence of AT2 receptor antagonist (PD 123319) (upper panel). 15 µL PBS with or without VEGF (100 ng/mL) or PD 123319 (50, 250 µg/mL) was injected intradermally. Relative ratios of Evans blue dye content of the leaky skin (mean ± SD, n = 6) are plotted (bottom panel). *P < 0.05.
We further assessed the effect of AT1 receptor signal for vascular permeability by using AT1a receptor–deficient mice (AT1a KO) (supplemental Figure I, available online at http://atvb.ahajournals.org). In the Miles assay, the extravasations of dye induced by VEGF were apparently reduced in AT1a KO mice compared with that in wild-type mice (41.75 ± 27.03 versus 12.61 ± 5.62 ng/mg tissue, respectively, \( P < 0.05 \)). The difference between WT and AT1a KO mice was not statistically significant.

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**Effect of Inflammatory Stimulation**

Although ARB decreased the VEGF-induced vascular permeability in vivo, the effect of ARB on leakage attributable to inflammation was not clear. Therefore, we investigated whether ARB could inhibit vascular leakiness by inflammatory mediators. We used mustard oil to stimulate inflammation in the ear skin of wild-type mice and AT1a KO mice. As shown in Figure 3, the extravasation of Evans blue dye was visibly increased in the ear skin of wild-type mice treated with mustard oil compared with the untreated group. On the other hand, such extravasation of Evans blue dye was similarly induced in AT1a KO mice as observed in wild-type mice. To further clarify the effect of other inflammatory mediators, we used histamine in the Miles assay (supplemental Figure II). Although ARB inhibited VEGF-induced vascular permeability, the extravasation of Evans blue induced by histamine was not inhibited by ARB. These results suggested that ARB specifically suppresses VEGF-induced vascular leakage.

**Effect of ARB to Endothelial Cell Junction**

Furthermore, we examined the expression of cell–cell junction protein, VE-cadherin, and ZO-1 in the HMVECs (Figure 4). In control monolayers of HMVECs, VE-cadherin, an adherence junction protein, was primarily found at cell–cell junctions (Figure 4A). On the other hand, on stimulation with VEGF, significant loss of VE-cadherin expression (Figure 4B) and some gaps between cells (Figure 4B, arrowheads) were observed. ARB pretreatment prevented the loss of VE-cadherin expression and maintained cell–cell junction integrity (Figure 4C, D).

**Figure 3.** Response of AT1 receptor signaling pathway in plasma leakage induced by inflammation. Representative photographs of ears from wild-type (WT) and AT1a KO mice after treatment with mustard oil (5% in mineral oil) (left panels). The quantities of extravasated Evans blue in mouse ears (mean ± SD, n = 5 or 6) are plotted (right panel). \( * P < 0.05 \). The difference between WT and AT1a KO mice was not statistically significant.

**Figure 4.** Effect of VEGF and ARB on endothelial junction protein, VE-cadherin and ZO-1. VE-cadherin expression (A-D) and ZO-1 expression (E-H) were studied in HMVEC monolayers. (A,E) HMVECs cultured in normal condition. (B,F) HMVECs cultured and stimulated with VEGF (50 ng/mL) for 60 minutes. (C,G) HMVECs pretreated with ARB (3 \( \times 10^{-6} \) M) for 20 minutes and subsequently exposed to VEGF (50 ng/mL). (D,H) HMVECs treated with ARB (3 \( \times 10^{-6} \) M) alone. Right panel in A-D shows high power view indicated by box. Arrow heads in (B) indicate the gaps between adjacent ECs and lack of VE-cadherin. Arrow heads in E, G, and H indicate accumulation of ZO-1 protein in the cell to cell junction. Nuclei were labeled with Hoechst 33258. Bar in A indicates 40 μm (left panel in A-D) and 15 μm (right panel in A-D) and bar in E indicates 30 μm (E-H).
were observed as previously reported. However, ARB inhibited VEGF-mediated loss of VE-cadherin expression and disorganization of junctions (Figure 4C). Moreover, as observed in VE-cadherin expression, ARB inhibited VEGF-mediated loss of ZO-1 (a tight junction protein) expression (Figure E-H). These data suggested that ARB reduces VEGF-mediated hyperpermeability through the prevention of disorganization of junction proteins at endothelial cell gaps.

**VEGF Gene Therapy With ARB in Ischemic Model**

At present, VEGF administration is thought to be a promising approach for managing ischemic patients. However, although VEGF induces angiogenesis in ischemic lesions, it also induces vascular leakage which results in edema. We investigated whether VEGF-induced edema was suppressed by ARB in a leg ischemia model of mice. Firstly, we determined whether transfer of naked plasmid containing VEGF DNA promoted hindlimb angioedema in normal mice and whether ARB could inhibit such edema (Figure 5). Naked plasmid expressing VEGF under the control of the CMV promoter (pSh-VEGF) or naked plasmid expressing LacZ (CMV promoter) (pSh-LacZ) was injected directly into the gastrocnemius muscle on day 0 and ARB or equivalent volume of DMSO (as a negative control) was injected intravenously on every other day (Figure 5A). After 20 days of injection of plasmid, the hindlimbs injected with pSh-VEGF showed abundant edematous free space in the gastrocnemius muscles in the case of DMSO injected group. Conversely, injection of pSh-LacZ as a control plasmid did not lead to the formation of such edematous space (Figure 5B). In the case of ARB-treated group, the edematous free space induced with pSh-VEGF was not observed and was similar to the DMSO treated group. We also measured the circumference of the hindlimbs, which reflects the degree of angioedema (supplemental Figure III). As observed in the sections of the gastrocnemius muscle, increase in circumference of the hindlimbs induced by pSh-VEGF injection was suppressed by ARB treatment.

Based on these experiment, we confirmed that VEGF-induced angioedema was inhibited by ARB. Next we carried out the VEGF gene therapy in the hindlimb ischemia model with ARB. The experimental schedules are shown in Figure 6A. As observed in normal mice injected with VEGF expression plasmid and ARB, VEGF-mediated edema estimated by hindlimb circumference in ischemia model was inhibited by ARB administration (Figure 6B). Next, we determined the effect of ARB treatment on thin capillary growth (Figure 6C),
because AT1 receptor signaling might have a function in angiogenesis in vivo. Vascular density and edematous free space formation in ischemia-induced gastrocnemius muscle were enhanced by pSh-VEGF injection compared with limbs injected with pSh-LacZ. Of note, pSh-VEGF injection with ARB enhanced thin capillary blood vessel formation in ischemia-induced gastrocnemius muscle as did pSh-VEGF injection alone; however, angioedema was suppressed by simultaneous administration of ARB. During collateral growth, deposition of fibronectin provides a scaffold for the migration of smooth muscle cells. ARB does not appreciably influence fibronectin leakage (supplemental Figure IV). These observations suggested that ARB suppresses VEGF-induced edema without inhibiting angiogenesis in the hindlimb ischemia model.

**Discussion**

Ang1 has been reported to protect vascular leakage; however, molecules regulating antipermeability by Ang1 have not been clearly identified. To address this issue, using DNA microarray analysis, we have identified genes whose expression is affected by Ang1 through Tie2 activation on ECs. Among numerous genes identified, we found that ACE was downregulated on the stimulation of Ang1 (data not shown). ACE converts angiotensin I to AII, and has been reported to induce permeability in in vitro analysis. Therefore, in the present study, we investigated the function of the receptor for AII in the regulatory mechanism of blood vessel permeability. We ascertained that blockade of AT1 receptor by ARB, but not antagonism of the AT2 receptor, resulted in inhibitory effects against VEGF-induced vascular hyperpermeability, in vivo. As many reports have indicated that there are reciprocal effects between AT1 and AT2 receptors in some cases, eg, cell proliferation, apoptosis, angiogenesis, and various physiological functions. The results of this study are conceptually consistent with previous reports.

Using AT1a KO mice, we found that AT1 receptor signal is involved in VEGF-mediated plasma leakage but not in inflammation-mediated permeability. These findings suggested that VEGF-induced vascular permeability is closely associated with the AII/AT1 receptor signaling pathway. In our analysis, eNOS phosphorylation by VEGF was inhibited by ARB, resulting in a decrease of NO release from HUVECs (data not shown). NO has been reported to be produced by VEGF through PI3K/Akt (PKB) pathway and to disrupt both cytoskeletal protein complex formation in mesenchymal cells and the rearrangement of the actin filament. Akt-deficient mice have also impaired VEGF-induced vascular permeability. Akt phosphorylation by VEGF was also inhibited by ARB (data not shown). Of interest, it has been suggested that Akt phosphorylation induces vascular leakage mediated by the regulation of cell–cell junction protein, ZO-1, occluding, and VE-cadherin in this issue (Figure 4). Taken together with our present results, we propose the possibility that NO release from ECs by VEGF receptor activation is promoted coordinately with AT1 receptor and both NO and activated Akt cause leakage at the cell–cell junction structure. However, Akt is involved in the proangiogenic property. Further precise analysis of Akt pathway by ARB will be necessary.
Histamine-induced leakage occurs through the H1 receptor and depends on increase in the cytoplasmic calcium concentration, and this causes a calcium/calmodulin-dependent activation of the myosin light chain kinase, resulting in promotion of actin–myosin interaction. The mechanisms of histamine-induced vascular leakiness are completely different from that induced by VEGF. Although we confirmed that expression of ACE is negatively regulated by the Ang1/Tie2 system and VEGF-mediated hyperpermeability was inhibited by blockade of ARB, it was reported that both VEGF and inflammatory stimuli–induced vascular permeability were suppressed in the dermis of mice harboring Ang1-overexpressing transgene in the keratinocytes. Therefore, there should be other pathways of negative regulation for vascular permeability induced by inflammation downstream of the Ang1/Tie2 system. As far as we examined, Ang1 inhibited VEGF induced vascular leakage rapidly as a protein level in a Miles assay (data not shown). Because the regulation of gene expression takes several hours, Ang1 might have another mechanism to suppress the edema along with ACE suppression. As it has been reported that Tie2 activation regulated cell adhesion between ECs and MCs through integrin activation,1,10 it is possible that tight cell adhesion between ECs and MCs also suppresses VEGF mediated vascular leakage mechanically.

It was reported that in mice deficient in placental growth factor (PIGF), which is a homolog of VEGF and binds to VEGFR-1, vascular leakage was induced by histamine but not by VEGF.30 This suggested that both of PIGF and VEGF are necessary for VEGF-mediated vascular hyperpermeability. Therefore, it is possible that cross-talk of receptors such as VEGFR-1, VEGFR-2, and AT1 might be essential for VEGF-induced hyperpermeability.

At present, by using the proangiogenic effects of VEGF, therapeutic angiogenesis in ischemic patients has started. In the present experiment, we performed local expression of VEGF using an expression plasmid system in the hindlimb, because administration of adenoviruses harboring VEGF gene accumulates in the liver causing systemic effects in the whole body. Our data clearly show that VEGF-mediated vascular leakage was inhibited by simultaneous injection of ARB in ischemic areas. Although Sasaki et al have reported that neovessel formation in response to ischemia was impaired in AT1aKO mice and ARB administrated in wild-type mice,23 we showed that ARB does not inhibit VEGF-induced angiogenesis in ischemic hindlimbs. Sasaki et al observed that the source of VEGF in ischemic hindlimb was transmigrating inflammatory cells into muscle and that the number of such migrating inflammatory cells in AT1aKO mice was fewer compared with that in wild-type mice.23 These results are consistent with our results, because ARB suppresses vascular leakiness and may inhibit transmigration of leukocyte from capillary into the ischemic muscle. In our experiments, we induced VEGF expression exogenously in ischemic hindlimbs. Therefore, VEGF could promote neovessel formation in the absence of inflammatory cells migrating into ischemic muscle.

For the further improvement of therapeutic angiogenesis, antagonists of AT1 receptor might be used to reduce edema, which is a side effect of therapy by VEGF gene injection or/and native VEGF protein administration. Moreover, ARB may be useful for reducing microvascular leakiness in diseases in which permeability results from elevated VEGF.

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Disclosures
None.

References


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**Supplemental Figure I.** Vascular leakage in AT1a receptor-deficient mice (AT1a KO; grey box) and C57 B/6 mice (WT; closed box). Quantitative analysis of extravasation of Evans blue dye (mean ± SD, n=3). *P < 0.05.

**Supplemental Figure II.** The graph of the histamine-induced vascular leakage affected by ARB in the Miles assay. 15 µl PBS with or without Histamine (100 µmol/L) or ARB (50, 250 µg/ml) was intradermally injected. The amounts of extravasated Evans blue in mouse skin (mean ± SD) were plotted. Differences between test groups were not statistically significant.
**Supplemental Figure III.** Measurement of hindlimb circumference. Experimental protocol was the same as described in Figure 5. Circumference of the hindlimbs was measured at a point 1 cm from the knee. Values are means ± SD; n = 4 mice per group. *P* < 0.05 comparing pSh-VEGF injected group and pSh-VEGF+ARB treated group.

**Supplemental Figure IV.** Representative photographs of anti-fibronectin antibody staining of mice skeletal muscle injected with plasmid DNA. Experimental protocol was the same as described in Figure 5. Brown color indicates fibronectin-staining. Arrowheads show the accumulation of fibronectin-positive area around hindlimb blood vessels. Bars indicate 50 μm.