Angiotensin II Type 1 Receptor–Mediated Inflammation Is Required for Choroidal Neovascularization

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Background—Choroidal neovascularization (CNV) is a critical pathogenesis in age-related macular degeneration, the most common cause of blindness in the developed countries. The aim of the current study was to determine the involvement of the renin-angiotensin system (RAS) with the development of CNV, using human surgical samples and the murine model of laser-induced CNV.

Methods and Results—In the human and murine CNV tissues, the vascular endothelium expressed angiotensin II type 1 receptor (AT1-R), AT2-R, and angiotensin II. The CNV volume was significantly suppressed by treatment with an AT1-R blocker telmisartan, but not with an AT2-R blocker. AT1-R signaling blockade with telmisartan inhibited various inflammatory mechanisms including macrophage infiltration and upregulation of VEGF, intercellular adhesion molecule-1 (ICAM-1), MCP-1, and IL-6 in the retinal pigment epithelium-choroid complex. A PPAR-γ antagonist partially but significantly reversed the suppressive effect of telmisartan on in vivo induction of CNV and in vitro upregulation of ICAM-1 and MCP-1 in endothelial cells and IL-6 in macrophages, showing the dual contribution of PPAR-γ-agonistic and AT1-R-antagonistic actions in the telmisartan treatment.

Conclusions—AT1-R–mediated inflammation plays a pivotal role in the development of CNV, indicating the possibility of AT1-R blockade as a novel therapeutic strategy to inhibit CNV. (Arterioscler Thromb Vasc Biol. 2006;26:2252-2259.)

Key Words: choroidal neovascularization ■ inflammation ■ intercellular adhesion molecule-1 ■ renin-angiotensin system ■ vascular endothelial growth factor

Age-related macular degeneration (AMD) is the most common cause of blindness in the developed countries.1 The macula is located at the center of the retina and the visual acuity depends on the function of the macula where cone photoreceptors are abundant. AMD is complicated by choroidal neovascularization (CNV), leading to severe vision loss and blindness. During CNV, new vessels from the choroid invade the subretinal space, resulting in the formation of neovascular tissues including vascular endothelial cells, retinal pigment epithelial cells, and macrophages.2 Bleeding and lipid leakage from the immature vessels cause the damage to the retinal functions.

Molecular and cellular mechanisms in the development of CNV are not fully elucidated. CNV seen in AMD develops after chronic inflammation adjacent to the retinal pigment epithelium, Bruch’s membrane, and choriocapillaris. Vascular endothelial growth factor (VEGF) plays a pivotal role in the development of CNV.3-5 VEGF is expressed in macrophages and retinal pigment epithelial cells in the experimental model of laser-induced CNV3 and surgically excised human neovascular tissues.4 Blockade of VEGF signaling caused significant suppression of experimental CNV.5 A recent clinical trial showed that the intravitreal administration of a VEGF antagonist ameliorated the visual outcome as compared with sham injections.6 Inflammatory processes, including macrophage infiltration7,8 and cytokine network,5,9 play crucial roles in CNV, as well as pathological neovascularization seen in solid tumor. Choroidal neovascular tissues express inflammation-related adhesion molecules including intercellular adhesion molecule (ICAM)-1 in both human surgical specimens10 and the laser-induced murine model.11

The renin-angiotensin system (RAS) plays an important role in the regulation of systemic blood pressure. Angiotensin II (Ang II), the final product of the system, has 2 cognate receptors, Ang II type 1 receptor (AT1-R) and AT2-R.12 Because the major pathogenic signaling of Ang II is mediated by AT1-R, AT1-R blockers (ARBs) are widely used for patients with hypertension. Recently, various functions of the RAS have been pointed out, including angiogenesis, inflammation, and tumor growth.13-16 We have recently shown the inhibitory effect of an ARB on several retinal pathologies mediated by ICAM-1, including ischemia-induced retinal
neovascularization and endotoxin-induced retinal inflammation. Although treatment with losartan, one of ARBs, was reported to suppress experimental CNV, the mechanism of the ARB-induced effect remains to be clarified. Here we show the molecular and cellular mechanisms by which the RAS plays critical roles in the development of CNV, using human surgical samples and the experimental model of laser-induced CNV.

Materials and Methods

Animals
C57BL/6 mice (7 to 10 weeks old) (CLEA, Tokyo, Japan) were utilized. All animal experiments were conducted in accordance with the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of CNV
Laser-induced CNV is widely used as an animal model for neovascular AMD and reflects the pathogenesis of choroidal inflammation and neovascularization seen in AMD. In this model, new vessels from the choroid invade the subretinal space after photoocoagulation. Laser photoocoagulation was performed around the optic nerve using a slit lamp delivery system (NOVUS spectra; Japan Lumenis, Tokyo, Japan), as described previously.

Treatment With an AT1-R or AT2-R Blocker
Animals were pretreated with intraperitoneal injections of an ARB (telmisartan or valsartan), an AT2-R blocker (PD123319; Sigma, St. Louis, Mo), or phosphate-buffered saline daily for 5 days before photoocoagulation and the treatment were continued daily until the end of the study. Telmisartan and valsartan were kind gifts of Boehringer Ingelheim (Ingelheim, Germany) and Novartis Pharma (Basel, Switzerland), respectively. Telmisartan was injected to mice with the dose of 0.5 or 5 mg/kg body weight and valsartan was injected with the dose of 10 mg/kg body weight. PD123319 was administered to mice at the dose of 1, 10, or 30 mg/kg body weight.

Quantification of Laser-Induced CNV
One week after laser injury, eyes were enucleated and fixed with 4% paraformaldehyde. Eye cups obtained by removing anterior segments were incubated with 0.5% fluorescein isothiocyanate-isocitelic B4 (Vector Laboratories, Burlingame, Calif). CNV was visualized with blue argon laser wavelength (488 nm) using a scanning laser confocal microscope (FV1000; Olympus, Tokyo, Japan). Horizontal optical sections of CNV were obtained every 5 μm step of CNV, and area-adjusted number of macrophages per 10 000-μm² area of CNV was calculated.

Immunohistochemistry for AT1–R, AT2–R, Ang II, CD68, and PECAM-1
Immunohistochemical experiments were performed for human and murine CNV. Human CNV tissues were obtained from patients with AMD who gave their informed consent to our study, during vitrectomy performed at Keio University Hospital. The protocol of the study adhered to the tenets of the Declaration of Helsinki. Immunohistochemical analyses for AT1-R, AT2-R, or Ang II were performed, as described previously.

Immunohistochemical analyses for infiltrating macrophages, human paraffin sections were incubated with rabbit polyclonal antibodies against human CD68 and AT1-R (Santa Cruz Biotechnology). Avidin-Alexa 488-tagged and Avidin-Alexa 546-tagged secondary antibodies (Molecular Probes, Eugene, Ore) were then applied.

Quantification of Infiltrating Macrophages
Three days after laser injury, eyes were enucleated and whole-mount choroid-sclera complexes were incubated overnight at 4°C with a goat polyclonal antibody against mouse PECAM-1 (CD31) and a rat polyclonal antibody against F4/80 (Serotec, Oxford, UK). Avidin-Alexa 488-tagged and Avidin-Alexa 546-tagged secondary antibodies were then applied for 2 hours at room temperature, and CNV was viewed with the scanning laser confocal microscope. PECAM-1-stained area of CNV and F4/80-positive macrophages were evaluated in every 5-μm step of CNV, and area-adjusted number of macrophages per 10 000-μm² area of CNV was calculated.

Reverse-Transcription Polymerase Chain Reaction Analyses for the RAS Components and Various Inflammation-Related Molecules
Total RNA was isolated from the retinal pigment epithelium (RPE)-choroid complex 3 days after photoocoagulation. Western blot analyses for AT1-R, AT2-R, or Ang II were performed, as described previously.

Enzyme-Linked Immunosorbent Assay for VEGF, VEGFR-1, VEGFR-2, ICAM-1, MCP-1, and IL-6
The RPE-choroid complex was carefully isolated from the eyes 3 days after photoocoagulation and placed into 200 μL of lysis buffer supplemented with protease inhibitors and sonicated. The lysate was centrifuged at 15 000 rpm for 15 minutes at 4°C and the levels of VEGF, VEGF receptor (VEGFR)-1, VEGFR-2, ICAM-1, monocyte chemotactic protein (MCP)-1, and IL-6 were determined with the mouse VEGF, VEGFR-1, VEGFR-2, ICAM-1, MCP-1, and IL-6 enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, Minn) according to the manufacturer’s protocols.

Blockade of PPAR-γ Signaling
Telmisartan has recently been characterized as a partial agonist of a steroid-type nuclear receptor, peroxisome proliferator-activated receptor (PPAR)-γ, a known negative regulator of inflammation and angiogenesis. To evaluate whether ARBs suppress CNV via PPAR-γ pathway, we simultaneously administered one of the ARBs and a selective PPAR-γ antagonist, GW9662 (Allexis Biochemicals, San Diego, Calif), GW9662 was administered at the dose of 0.1 or 1 mg/kg body weight simultaneously with telmisartan at the dose of 5 mg/kg body weight or valsartan at the dose of 10 mg/kg body weight.

In Vitro Assays
Murine brain-derived capillary endothelial cells (bEnd3) were incubated with DMEM (Sigma) containing tumor necrosis factor (TNF)-α (Sigma; 5 ng/mL) alone, TNF-α plus telmisartan (300 nM), or TNF-α plus telmisartan and GW9662 (1 or 10 μmol/L) for 2 hours and total cellular RNA was processed for reverse-transcription (RT)-PCR analyses for ICAM-1, MCP-1, and VEGFR-1. For protein analyses, supernatant and cell lysates were collected after 6-hour incubation and then the concentration of MCP-1 in the supernatant and ICAM-1 in the cell lysates were measured by the ELISA kits (R&D Systems). Murine macrophages (RAW264.7) were treated with DMEM containing lipopolysaccharide (LPS) (200 ng/mL) alone, LPS plus telmisartan (300 nM), or LPS plus telmisartan and GW9662 (1 or 10 μmol/L). After 6-hour incubation, the supernatant and total cellular RNA were processed for ELISA and RT-PCR analyses for VEGF and IL-6.
Morphometric and Statistical Analyses

All results were expressed as mean±SD. The values were processed for statistical analyses (Mann-Whitney test). Differences were considered statistically significant when P<0.05.

Results

Tissue Localization and Upregulation of the RAS Components in CNV

Immunohistochemistry for the RAS components was performed to identify their expression on the human and murine CNV. In the CNV tissues from patients with AMD, the vascular endothelium was positive for AT1-R (Figure 1A), AT2-R (Figure 1B), and Ang II (Figure 1C). Similarly, the murine model of CNV showed AT1-R (Figure 1D), AT2-R (Figure 1E), and Ang II (Figure 1F) staining on PECAM-1 (CD31)-positive endothelial cells. Additionally, mRNA expression of AT1-R, AT2-R, and angiotensinogen was higher in the RPE-choroid complex of laser-treated mice than in age-matched normal controls (Figure 1G). Similarly, AT1-R, AT2-R, and Ang II protein levels were significantly increased by inducing CNV (P<0.05) (Figure 1H and 1I).

Suppression of CNV with AT1-R, but Not AT2-R, Blockade

The CNV volume was measured to evaluate the effects of the RAS on the development of CNV. CNV was significantly suppressed by blocking AT1-R, but not AT2-R, signaling in a dose-dependent manner. Telmisartan-treated mice at the dose of 5 mg/kg showed a significant (P<0.001) decrease in the CNV volume (226 925±44 069 µm³), compared with vehicle-treated mice (542 041±96 231 µm³) (Figure 2A and 2C). However, AT2-R blockade with PD123319 did not significantly change the CNV volume (P>0.05), compared with vehicle-treated animals (Figure 2B and 2C).

Effects of Telmisartan on Macrophage Infiltration

We immunohistochemically analyzed the infiltration of macrophages in human (Figure 3A) and murine (Figure 3B) CNV. CD68-positive macrophages, also bearing AT1-R, were present in the human CNV tissues (Figure 3A). Additionally, we revealed that F4/80- and AT1-R–positive macrophages were associated with murine CNV tissues (Figure 3B). Telmisartan-treated mice showed a significant decrease in the number of F4/80-positive macrophages, compared with vehicle-treated animals (P<0.05) (Figure 3C and 3D).
Inhibition of Angiogenic and Inflammatory Molecules by AT1-R Blockade

To determine whether AT1-R blockade affects angiogenic and inflammatory molecules related to the pathogenesis of CNV, mRNA expression of VEGF, VEGFR-1, VEGFR-2, ICAM-1, MCP-1, IL-6, TNF-α, and cyclooxygenase (COX)-2 in the RPE-choroid complex was analyzed by semi-quantitative RT-PCR (Figure 4A). mRNA expression of VEGF, VEGFR-1, VEGFR-2, ICAM-1, MCP-1, IL-6, TNF-α, and COX-2 in the RPE-choroid complex were upregulated by inducing CNV. AT1-R blockade by systemic administration of telmisartan substantially reduced mRNA expression of VEGF (both 164 and 121), VEGFR-1, ICAM-1, MCP-1, and IL-6. Additionally, protein levels of VEGF, VEGFR-1, VEGFR-2, ICAM-1, MCP-1, and IL-6 in the RPE-choroid complex were higher in mice with CNV than in age-matched normal controls (Figure 4B to 4G). AT1-R blockade by telmisartan significantly suppressed protein levels of VEGF, VEGFR-1, ICAM-1, MCP-1, and IL-6.

AT1-R-Blocking and PPAR-γ-Activating Effects of Telmisartan In Vivo

Recent reports have demonstrated that telmisartan, unlike other ARBs, serves as a partial agonist for PPAR-γ.20,21 We examined whether ARBs suppress CNV via PPAR-γ signaling. Mice receiving telmisartan at the dose of 5 mg/kg or valsartan at the dose of 10 mg/kg, both of which significantly suppressed CNV, were simultaneously administered with a selective PPAR-γ antagonist, GW9662 (Figure 5). Telmisartan-induced suppression of CNV (226,925±44,069 μm²) was partially (22%) but significantly (P<0.01) reversed by the simultaneous administration of a higher dose (1 mg/kg) of GW9662 (295,521±46,311 μm²). The reversed level was still significantly lower than the vehicle-treated level (P<0.01). On the contrary, a high dose of GW9662 did not change the valsartan-induced effect (P>0.05).

AT1-R–Blocking and PPAR-γ–Activating Effects of Telmisartan In Vitro

To confirm the telmisartan-induced suppression of choroidal expression of various inflammatory and angiogenic molecules in vivo (Figure 4), we further performed in vitro analyses (Figure 6). We analyzed mRNA (Figure 6A) and protein (Figure 6B and 6C) levels of ICAM-1, MCP-1, and VEGFR-1 in b-End3 vascular endothelial cells. We also examined mRNA (Figure 6D) and protein (Figure 6E and 6F) levels of VEGF and IL-6 in RAW264.7 macrophages.

b-End3 cells were cultured with TNF-α alone, TNF-α plus telmisartan, or TNF-α plus telmisartan and GW9662 (1 or 10 μmol/L). mRNA (Figure 6A) and protein levels (Figure 6B and 6C) of ICAM-1 and MCP-1, strongly induced by the exposure to TNF-α, were significantly suppressed by the treatment with telmisartan. Telmisartan-induced reduction of ICAM-1 and MCP-1 levels were partially but significantly reversed by the simultaneous administration of GW9662 (P<0.05). VEGFR-1 mRNA and protein levels show no significant changes in the in vitro assays (data not shown).

RAW264.7 cells were cultured with LPS alone, LPS plus telmisartan, or LPS plus telmisartan and GW9662 (1 or 10 μmol/L). mRNA (Figure 6D) and protein (Figure 6E and 6F) levels of VEGF and IL-6, strongly induced by LPS stimulation, were significantly suppressed by treatment with telmisartan (P<0.01). Administration of GW9662 partially but significantly reversed the suppressive effect of telmisartan on IL-6, but not VEGF, production (P<0.05).

Discussion

The present study reveals, for the first time to our knowledge, several important findings concerning the relationship of the RAS with CNV. First, the RAS components were present in human and murine CNV, and upregulated when experimental CNV was induced (Figure 1). Second, CNV was suppressed by blocking AT1-R, but not AT2-R, signaling (Figure 2). Third, the cellular and molecular mechanisms in the ARB-induced suppression of CNV included the inhibitory effect on macrophage infiltration (Figure 3) and on expression of inflammation-related molecules including chemotactants, adhesion molecules and cytokines in the RPE-choroid complex (Figure 4). Additionally, we demonstrate the PPAR
γ-mediated effect of telmisartan on CNV in vivo (Figure 5) and on inflammatory responses in vitro (Figure 6).

We revealed that Ang II and its receptors AT1-R and AT2-R were detected on microvessels in the human and murine CNV tissues (Figure 1), showing the existence of the ligand-receptor system in CNV. Moreover, these molecules were upregulated by inducing CNV. Ang II-mediated AT1-R signaling contributes to pathological conditions with inflammation and neovascularization, including atherosclerosis and tumor. On the other hand, AT2-R signaling leads to suppression of cell growth. Whereas normal adult animals have low AT2-R expression, vascular injury and exposure to cytokines augment AT2-R levels.

In the present study, blockade of AT1-R (Figure 2A), but not AT2-R (Figure 2B), led to significant suppression of CNV. Recent studies have revealed that Ang II-induced neovascularization was suppressed by blocking AT1-R signaling. In vitro, Ang II-induced VEGF expression in vascular smooth muscle cells via AT1-R. In the models of tumor and myocardial ischemia, AT1-R–deficient mice showed suppression of neovascularization as well as inflammatory responses. However, AT2-R signaling is suggested to play a minimal role in pathological angiogenesis, at least in several in vivo models, including the current laser-induced CNV. The present study, in accordance with our recent data on the ARB-induced suppression of pathological retinal neovascularization, indicates the possibility of AT1-R blockade as a useful strategy for the treatment of CNV.

As the cellular mechanism promoting CNV, our data show AT1-R expression in infiltrating macrophages in both human and murine CNV (Figure 3A and 3B) and the ARB-mediated suppression of macrophage infiltration as well as CNV (Figure 3C). Macrophages were detected in human and murine CNV tissues. The development of CNV has been...
shown to depend on macrophage infiltration, because mice receiving pharmacological deletion of macrophages\textsuperscript{7} and mice deficient in CCR-2, a receptor for MCP-1,\textsuperscript{8} exhibited suppression of CNV. One of macrophage roles in the development of CNV is to promote neovascularization by secreting VEGF at the lesion where RPE and vascular endothelial cells produce MCP-1 for macrophage recruitment.\textsuperscript{4} The recent findings\textsuperscript{28–30} that inflammatory cell adhesion to the retinal vasculature controls neovascularization provide new insights to therapeutic strategies for ocular neovascularization. Indeed, the anti-inflammatory effect of corticosteroid is currently utilized to treat AMD and diabetic retinopathy.\textsuperscript{31,32}

As the molecular mechanism promoting CNV, recent reports have pointed out the association of adhesion molecules,\textsuperscript{10,11} chemokines,\textsuperscript{8} and cytokines\textsuperscript{33} in addition to VEGF.\textsuperscript{3–6} The present study showed for the first time to our knowledge the ARB-induced suppression of various inflammation-related molecules, including VEGF, VEGFR-1, ICAM-1, MCP-1, and IL-6, which were upregulated after the induction of CNV (Figure 4). The current ARB-induced suppression of VEGF in CNV is supported by previous in vivo\textsuperscript{13} and in vitro\textsuperscript{14} studies. VEGFR-1 is expressed in inflammatory leukocytes including macrophages. The ARB-induced decrease in choroidal VEGFR-1 seen in the present study (Figure 4) is compatible with and explained at least in part by the suppression of VEGFR-1-bearing macrophage infiltration. Indeed, our in vitro experiments (Figure 6) showed no remarkable change in endothelial VEGFR-1 expression following the ARB treatment (data not shown). Additionally, we performed in vitro experiments showing that ICAM-1 and MCP-1 levels in endothelial cells and VEGF and IL-6 levels in macrophages were significantly reduced by telmisartan (Figure 6). The present data on the ARB-mediated inhibition of various molecules related to the development of CNV in vivo (Figure 4) and in vitro (Figure 6) indicate the molecular mechanisms in the anti-angiogenic effects of AT1-R blockade by targeting multiple inflammatory steps.

Recent reports demonstrated that telmisartan, unlike other ARBs, ameliorated insulin resistance and lipid metabolism by functioning as a PPAR-\(\gamma\) partial agonist.\textsuperscript{20,21} In the present study, consistent with the previous findings, telmisartan-induced suppression of CNV was mediated in part by PPAR-\(\gamma\), because a PPAR-\(\gamma\) antagonist GW9662 significantly reversed the anti-angiogenic effect of telmisartan by 22\% in vivo (Figure 5). Moreover, we revealed that telmisartan-induced suppression of ICAM-1 and MCP-1 lev-
els in endothelial cells and IL-6 levels in macrophages were significantly reversed by the simultaneous administration of the PPAR-γ antagonist (Figure 6). Recently, several anti-pathogenic roles of PPAR-γ have been clarified. As for anti-angiogenic functions, a PPAR-γ ligand reduced laser-induced CNV,34 which is complementary to our in vivo experiments with PPAR-γ signaling blockade (Figure 5). As for anti-inflammatory functions, PPAR-γ signaling, exerting antagonistic action against nuclear factor (NF)-κB, inhibited the in vitro expression of IL-6 and IL-1β.35 In the myocardial ischemia-reperfusion model, PPAR-γ signaling ameliorated cardiac functions by inhibiting the expression of ICAM-1 and MCP-1 and subsequent leukocyte infiltration.36 Whereas ARBs have anti-angiogenic and anti-inflammatory effects by blocking AT1-R signaling, PPAR-γ ligands are similar to ARBs in these effects. The present study showed the suppressive effects of telmisartan on experimental CNV via these dual pathways.

Epidemiologic risk factors for AMD include hypertension,37 atherosclerosis,37 and dyslipidemia,38 all of which are the main components of the metabolic syndrome. The suppression of CNV shown in the present study is thought to be mediated by AT1-R blockade and PPAR-γ activation, both of which also ameliorate the pathogenesis of the metabolic syndrome, a systemic background underlying AMD. Currently, the treatments for AMD complicated by CNV include photodynamic therapy39 and anti-VEGF therapy.40 Because their therapeutic intervention follows the advanced stage when the visual function is irreversibly affected, alternative early treatment is required. Here we show the efficacy of ARBs, which have established their efficacy and safety, on CNV using the murine laser-induced model, which has proven to be a highly predictive preclinical platform for drug efficacy in patients. Reasonably, AT1-R blockade, which not only inhibits inflammatory neovascularization in the eye but also improves the systemic background, is likely to be a novel therapeutic strategy as a preventive, early and additive treatment for AMD. A large-scale, prospective, and randomized clinical trial is awaited to validate the inhibitory effect of AT1-R blockade on CNV.

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