Suppression of Experimental Autoimmune Uveitis by Angiotensin II Type 1 Receptor Blocker Telmisartan

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PURPOSE. Angiotensin II type 1 receptor (AT1-R) blockers are used widely for the treatment of patients with hypertension. Recent reports have suggested that AT1-R also plays a key role in various inflammatory conditions. The aim of this study was to examine whether blockade of AT1-R is effective in the suppression of murine experimental autoimmune uveoretinitis (EAU).

METHODS. C57BL/6 mice were immunized with human interphotoreceptor retinoid binding protein–derived peptide 1–20 (hIRBP-p). Telmisartan, an AT1-R blocker, was administered daily by intraperitoneal injection. On day 21 after immunization, the severity of EAU was assessed clinically and histopathologically. With the use of flow cytometry, the activation of draining lymph node (LN) cells was assessed by cell proliferation response against hIRBP-p and by the number of CD44high activated CD4+ T cells present. In addition, mRNA expression of ICAM-1, MCP-1, and IFN-γ in the eye was analyzed by reverse-transcriptase PCR, and the number of retinal adherent leukocytes was counted by retinal perfusion labeling.

RESULTS. Telmisartan significantly suppressed EAU clinically and histopathologically. Intraocular mRNA expression of ICAM-1 and MCP-1 was downregulated, and the retinal adherent leukocytes were significantly decreased in telmisartan-treated mice compared with vehicle-treated mice. LN cell proliferative responses against hIRBP-p and the number of CD44highCD4+ T cells were remarkably reduced in telmisartan-treated mice.

CONCLUSIONS. Systemic administration of telmisartan significantly suppressed EAU by the inhibition of antigen-specific T-cell activation in the LNs and of leukocyte adherence in the retina. These results indicate that telmisartan may be a novel therapeutic regimen for patients with endogenous uveitis. (Invest Ophthalmol Vis Sci. 2009;50:2255–2261) DOI:10.1167/iovs.08-2649

Endogenous uveitis, represented by Behc¸et disease, Vogt-Koyanagi-Harada disease (VKH), birdshot retinochoroidopathy, and sarcoidosis, is a sight-threatening ocular inflammatory disease.1 Although endogenous uveitis covers a range of different clinical entities, some of them are thought to have an autoimmune pathogenesis. Systemic and local administration of corticosteroids or immunomodulatory agents, or both, are the major treatments for these diseases, and recently infliximab, an anti-TNF-α antibody, has been shown to be effective for Behc¸et disease with uveitis. However, serious side effects are not negligible.2–4 Patients who cannot take medications because of the side effects or patients who are not responsive to the existing medications experience unavoidable impaired visual function. Thus, it is necessary to find new medications that are effective for endogenous uveitis but have less severe side effects than existing medications.

Although the renin-angiotensin system (RAS) plays an important role in the regulation of blood pressure, recent studies have demonstrated that RAS is also involved in immune function. RAS is expressed on inflammatory cells,5,6 and signals through RAS stimulate the accumulation of neutrophils,7 accelerate the differentiation of dendritic cells (DCs),8 and promote the production of inflammatory chemokines by vascular endothelial cells.9 Angiotensin II (AngII) is an effector molecule of RAS. AngII has two distinct subclasses of receptors, angiotensin II type 1 receptor (AT1-R) and AT2-R,10 in which AT1-R mediates major pathogenic signals of AngII. AT1-R blockers (ARBs) are thought to act not only as antihypertensive but also as immunosuppressive agents. In previous reports, AngII was suggested to be involved in the pathogenesis of diabetic retinopathy,11 and diabetes-induced retinal inflammation was reduced by blocking RAS.12 Inflammation through AT1-R was required for retinal13 and choroidal14 vascularization. With regard to inflammatory disease, ARBs have been reported to be effective in suppressing the inflammation of collagen-induced arthritis (CIA).15,16 In addition, we and others demonstrated previously that endotoxin-induced uveitis (EUU) was ameliorated by the inoculation of ARBs.17,18 EUU is induced by the administration of lipopolysaccharide (LPS), which leads to the direct activation of resident macrophages that mediate the infiltration of neutrophils and mononuclear cells.19 Therefore, it has not been known whether autoimmune responses are involved in EUU. Experimental autoimmune uveoretinitis (EAU) is an animal model of endogenous uveitis that can be induced by the immunization of susceptible animals with interphotoreceptor retinoid-binding protein (IRBP), an eye-specific retinal antigen, or by the adoptive transfer of CD4+ T cells specific for retinal antigens.20 In the present study, we examined whether AT1-R blockade by telmisartan also has a suppressive effect on T cell–mediated autoimmune uveitis by using EAU.

METHODS

Mice, Reagents, and Monoclonal Antibodies

Seven- to 9-week-old female C57BL/6 mice were obtained from Japan CLEA (Shizuoka, Japan). The mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. High-pressure liquid chromatography-purified human IRBP peptide 1–20 (hIRBP-p) was purchased from Takara (Shiga, Japan).
Complete Freund adjuvant (CFA) and *Mycobacterium tuberculosis* H37Ra were purchased from Difco (Detroit, MI), and purified *Bordetella pertussis* toxin (PTX) was purchased from Sigma-Aldrich (St. Louis, MO). Telmisartan was a gift from Boehringer Ingelheim (Ingelheim, Germany).

**Induction and Scoring of EAU and AT2R1 Treatment**

hIRBP-p (200 μg) was emulsified in CFA (1:1 wt/vol) containing 5 mg/mL *M. tuberculosis* H37Ra. A total of 200 μL of the emulsion was injected subcutaneously into the neck. Concurrent with immunization, 1 μg PTX was injected intraperitoneally. Indicated doses of telmisartan or vehicle (2% or 4% dimethyl sulfoxide in phosphate-buffered saline [PBS]) were injected intraperitoneally daily from day −1 to the day before kill. Telmisartan- or vehicle-treated mice were clinically and histologically assessed for the severity of EAU on day 21 after immunization. Clinical score was graded on a scale of 0 to 4 in half-point increments, as described previously. For histologic assessment, enucleated eyes were fixed in 4% paraformaldehyde. Sections of samples were embedded in paraffin and stained with hematoxylin and eosin for pathologic study. The severity of EAU in each eye was scored on a scale of 0 to 4 in half-point increments according to a semiquantitative system described previously, and results are shown as mean scores of bilateral eyes of each mouse. Clinical and histopathologic assessments were performed by two ophthalmologists in a masked fashion. When the two observers had different scores, the mean value of each score was calculated.

**Cell Proliferation**

Draining lymph node (LN) cells were isolated and pooled on day 21 and cultured in triplicate in 96-well flat-bottomed microculture plates at a density of 6 × 10^5 cells/well in the presence or absence of the indicated dose of hIRBP-p. To assess proliferative responses, the cultures were pulsed with [3H]TdR (0.5 μCi/well) for the last 8 hours of a 72-hour culture and were harvested onto glass filters with an automated cell harvester. Radioactivity was measured by liquid scintillation spectrometry (Tomtec, Orange, CT), and the amount was expressed as cycles per minute.

**Delayed-Type Hypersensitivity Assay**

On day 20 after immunization, mice were injected intradermally with 10 μg/10 μL hIRBP-p suspended in PBS into the pinna of one ear. Ear swelling was measured after 24 hours with the use of a micrometer (Mitsutuyo, Tokyo, Japan). Antigen-specific delayed-type hypersensitivity (DTH) was measured as the difference in ear thickness before and after challenge. Results were expressed as specific ear swelling: (24-hour measurement − 0-hour measurement) for the tested ear − (24-hour measurement − 0-hour measurement) for the control ear.

**Lectin Labeling of Adherent Retinal Leukocytes**

The retinal vasculature and adherent leukocytes were imaged by perfusion labeling with rhodamine-coupled concanavalin A lectin (conA; Vector Laboratories, Burlingame, CA), as described previously. After deep anesthesia, the chest cavity was opened, and a 27-gauge cannula was introduced into the left ventricle. After injection of 2 mL PBS to remove erythrocytes and nonadherent leukocytes, 2 mL rhodamine-conjugated conA lectin was perfused through the left ventricle. After the eyes were enucleated, the retinas were flattened. Flatmounts were then imaged with the use of an epifluorescence microscope (IX71; Olympus, Tokyo, Japan), and the total number of conA-stained adherent leukocytes per retina was determined.

**Reverse Transcription–Polymerase Chain Reaction**

Total RNA was extracted from the eyes of EAU mice (day 21) or normal mice with an RNA isolation system (Iogen; Wako, Osaka, Japan) according to the manufacturer’s protocol. Complementary DNA was synthesized at 42°C for 2 hours in the presence of RNase H− reverse transcriptase and random primers (Superscript II; Invitrogen, Carlsbad, CA) in 20-μL reaction volumes. RT-PCR was conducted in a 50-μL reaction mixture using 1 μg cDNA as a template in the presence of 2.5 U Taq DNA polymerase (Takara). Sequences of sense and antisense primers and expected product sizes were as follows: ICAM-1 (505 bp), 5′-gtgctgcgttgtagggta and 3′-ctcggctggagactccagt; MCP-1 (292 bp), 5′-attcaaggtagctggagag and 3′-cagaggtctctgggttg; IFN-γ (364 bp), 5′-gtcctgctgacctgcctctctcagt and 3′-ggagctctggctgtggacctgg; and GAPDH (357 bp), 5′-gagggccctacatcggcttt and 3′-catcactccattcagccaggg. The thermal cycle consisted of denaturation at 94°C for 30 seconds; annealing at 57°C (ICAM-1 and GAPDH), 61°C (IFN-γ), and 65°C (MCP-1); and extension at 72°C for 45 seconds. Each cycle of amplification was repeated 35 times (PCR Thermal Cycler 480; Takara). Five microelectrodes of each PCR product was electrophoresed on a 2% agarose gel containing ethidium bromide in Tris-borate EDTA buffer and was photographed.

**Flow Cytometric Analysis**

Cervical and axillary LNs were harvested from 10 EAU and 10 normal mice and were pooled. First the cells were reprinted with unlabeled anti–CD16/32 monoclonal antibody (mAb) to avoid nonspecific binding of mAb to FcγR. Then they were incubated with FITC-conjugated anti–CD4 and PE-conjugated anti–CD44 mAb. After washing with PBS, the stained cells (live-gated based on forward and side scatter profiles and propidium iodide exclusion) were analyzed on a flow cytometer (FACSCalibur; BD Biosciences, Franklin, Lakes, NJ). Data were processed with the accompanying software (CellQuest; BD Biosciences) and expressed as mean fluorescence intensity.

**Measurement of Intraocular Inflammatory Cytokines and Chemokines with a Flow Cytometer**

We prepared intraocular protein extracts as follows: On day 21 after immunization, mouse eyes were enucleated bilaterally. Eyes from each mouse were homogenized in a 1.5-mL tube with a homogenizer after 100 μL PBS was added. Then the tubes were centrifuged at 15,000 rpm for 30 minutes, and the supernatants were kept at −80°C until use. We measured the concentrations of intraocular IFN-γ, IL-2, IL-6, and MCP-1 (Cytometric Beads Array Flex Set and FACSCalibur; BD Biosciences) according to the manufacturer’s protocol and used 50 μL undiluted protein extract from each sample.

**RESULTS**

**Telmisartan Suppression of EAU**

We evaluated clinical severity in telmisartan- and vehicle-treated EAU mice on day 21 after immunization. In telmisartan-treated mice, EAU was significantly suppressed compared with vehicle-treated mice clinically and histopathologically (Figs. 1A–C). In addition, though all the vehicle-treated mice showed histologic evidence of EAU, ocular inflammation was completely inhibited in some telmisartan-treated mice. Similar to the results of EAU, DTH measured by ear swelling after challenge with hIRBP-p was significantly suppressed in telmisartan-treated mice compared with vehicle-treated mice (Fig. 1D). No statistically significant difference was observed in clinical and histopathologic EAU severity and in DTH between telmisartan 5 mg/kg− and 2.5 mg/kg–treated mice. Therefore, mice treated with 2.5 mg/kg telmisartan were used for the further experiments.
Suppression of Intraocular Cytokine and Chemokine Production by Administration of Telmisartan

Given that EAU was suppressed in mice treated with telmisartan, we evaluated the mRNA expression of intraocular chemokines associated with leukocyte migration and adhesion by RT-PCR (Fig. 2). Systemic treatment with telmisartan markedly suppressed intraocular mRNA expression of ICAM-1 and MCP-1, whose levels of expression were elevated in vehicle-treated mice. Intraocular mRNA expression of IFN-γ was upregulated in vehicle-treated mice and reduced in telmisartan-treated mice. Next, we examined the intraocular production of IFN-γ, IL-6, IL-2, and MCP-1 at the protein level with the use of flow cytometry (Fig. 3). Consistent with the results of RT-PCR, the intraocular production of MCP-1 and IFN-γ was significantly decreased in telmisartan-treated EAU mice compared with that of vehicle-treated EAU mice. In addition, the intraocular production of IL-6 was suppressed in telmisartan-treated EAU mice. There was no difference in the concentration of IL-2 between the two groups (data not shown).

Effect of Telmisartan on Retinal Leukocyte Adhesion

Because ICAM-1 and MCP-1, which are associated with leukocyte adhesion and migration, were suppressed in telmisartan-treated mice, we examined whether leukocyte adhesion to the retina was suppressed in telmisartan-treated mice. For this purpose, retinal adherent leukocytes were imaged and counted according to a retinal perfusion labeling method with rhodamine-coupled conA. Leukocyte counts were evaluated in the posterior retina around the optic disc (Figs. 4A, 4D), the mid-peripheral retina near the equator of the globe (Figs. 4B, 4E), and the peripheral retina next to the ora serrata (Figs. 4C, 4F). Most adherent leukocytes were located in venules. Compared with vehicle-treated EAU mice (Figs. 4A–C), telmisartan administration suppressed leukocyte adhesion in the retina in all
Because the adoptive transfer of retinal antigen-specific T cells by Administration of Telmisartan

**Suppression of Activation of Lymph Node Cells by Administration of Telmisartan**

Because the adoptive transfer of retinal antigen-specific T cells can induce EAU, it has been suggested that EAU is a T cell-mediated disease. Therefore, we assessed whether the activation of hIRBP-p-specific T cells was also inhibited by the administration of telmisartan. We first analyzed the activation marker (CD44) on CD4+ T cells by flow cytometry. As shown in Figures 5A and 5B, both the expression and the number of CD44 on CD4+ T cells in the draining lymph nodes were significantly lower in telmisartan-treated EAU mice than in vehicle-treated EAU mice (24.6 ± 3.0 and 47.2 ± 9.4, respectively; Fig. 4G).

**FIGURE 2.** Effect of telmisartan on the expression of intraocular cytokines and chemokines during EAU. Mice were EAU induced and treated with vehicle or telmisartan (2.5 mg/kg) daily from day −1 to day 20. On day 21, ocular mRNA expression of ICAM-1, MCP-1, and IFN-γ was analyzed by RT-PCR. Systemic treatment with telmisartan markedly suppressed the mRNA expression of ICAM-1, MCP-1, and IFN-γ, whose expression was elevated in vehicle-treated mice. Representative images of each group are presented.

**FIGURE 3.** Intraocular concentrations of IFN-γ, IL-6, IL-2, and MCP-1 were measured with a flow cytometer on day 21. Concentration of (A) IFN-γ, (B) IL-6, and (C) MCP-1 were suppressed in telmisartan-treated mice compared with vehicle-treated mice. There was no difference in the concentration of IL-2 between the two groups (data not shown). *P < 0.05; **P < 0.001.

**DISCUSSION**

In this report, we demonstrated that telmisartan, an ARB, has a suppressive effect on an autoimmune animal model of uveitis, whose suppressive effects were indicated by inhibition of the adherence and migration of leukocytes in retinal vessels, down-regulation of inflammatory molecules in the eye, and insufficient activation of antigen-specific T cells in draining LNs.

In this study, IFN-γ, IL-6, MCP-1, and ICAM-1 were markedly downregulated in the eyes of telmisartan-treated EAU mice. AngII increases proinflammatory genes, including chemokines, cytokines, and adhesion molecules, under the control of NF-κB. These molecules were reported to be associated with the exacerbation of EAU. An adhesion pathway mediated by ICAM-1 and its ligand, leukocyte function-associated antigen-1, is important in various cell-to-cell interactions at inflammatory sites, and the administration of monoclonal antibodies to ICAM-1 is reported to suppress EAU. In addition, MCP-1 is upregulated by the induction of EAU and expressed by infiltrating leukocytes in the EAU retina. Furthermore, it is suggested that the ocular microenvironment loses its immunosuppressive properties because of local production of IL-6. One of the suppressive effects of telmisartan on EAU may be through the downregulation of these inflammatory molecules, which consequently suppresses the maturation and activation of T cells in the eye.

RAS exists in various tissues, including the reproductive tract, adrenal glands, thymus, and eye. Active renin, derived from the inactive precursor prorenin, cleaves the inactive substrate angiotensinogen to AngI, which is subsequently cleaved by angiotensin-converting enzyme (ACE) to form the effector molecule of the system, AngII. In the human eye, local production of angiotensinogen mRNA was reported in the retina, retinal pigment epithelium (RPE), and choroid, and its angiotensinogen protein was detected in the retina. Renin is also detected in human RPE and the choroid at the mRNA level. ACE is detected at the mRNA level in human RPE, choroid, and retina. In addition, ACE, AngII, AT-1R, and AT-2R were detected in murine and human retina at the protein level. Thus, local RAS exists in the eye and may contribute to the exacerbation of EAU. Considering that ocular tissues such as RPE produce inflammatory molecules under activation, telmisartan may participate in the suppression of EAU in part by suppression of the production of inflammatory molecules by ocular tissues.

In draining LNs, the proportion of CD44+CD4+ T cells decreased in telmisartan-treated EAU mice compared with
FIGURE 4. EAU-induced mice were treated with vehicle or telmisartan (2.5 mg/kg) daily from day −1 to day 6. On day 7, retinal adherent leukocytes were imaged by perfusion labeling with rhodamine-coupled concanavalin A lectin. One eye from each of five mice in each group was used. Representative images of flat-mounted retinas from (A–C) vehicle-treated mice and (D–F) telmisartan-treated mice (2.5 mg/kg) are presented. Images are around the optic disc (A, D), equator (B, E), and periphery (C, F). Systemic treatment with telmisartan led to the suppression of leukocyte adhesion. (G) Mean number of adherent retinal leukocytes per eye. Telmisartan-treated EAU mice showed significantly fewer adherent leukocytes than vehicle-treated mice. ***P < 0.001.

FIGURE 5. In vivo effect of telmisartan treatment in EAU. EAU-induced mice were treated with vehicle or telmisartan (2.5 mg/kg) daily from day −1 to day 21. LN cells were isolated 21 days after immunization. Five mice in each group were used. (A) Induction of CD44<sup>high</sup>CD4<sup>+</sup> T cells was determined by staining with anti-CD44 and anti-CD4 mAb and was expressed as the mean percentage of CD44<sup>high</sup>CD4<sup>+</sup> T cells. (B) The number of LN cells in each mouse was counted. (C) LN cells were cultured in the presence or absence of hIRBP-p. For estimating proliferation, 0.5 μCi [3H]dThd was added during the last 8 hours of a 72-hour culture. In the telmisartan-treated mice, percentage of CD44<sup>high</sup>CD4<sup>+</sup> T cells in LN, number of LN cells, and cell proliferation were significantly suppressed compared with vehicle-treated mice. *P < 0.05; **P < 0.01; ***P < 0.001.
vehicle-treated EAU mice. AngII was reported to accelerate the differentiation of DCs by stimulating the expression of costimulatory molecules on DCs. In addition, RAS was reported to be expressed on T cells and natural killer cells, and ARBs effectively suppressed the proliferation of anti-CD3-stimulated T cells. Therefore, the data from this study support our hypothesis that telmisartan can suppress the activation of T cells first through the inactivation of hIRBP-p-stimulated DCs and then through the direct inactivation of T cells in the draining LNs.

We evaluated retinal leukocyte counts on day 7 after immunization. It has been reported that ICAM-1 expression increased progressively during the development of EAU and was expressed predominantly in retinal venules, the sites of blood-retinal-barrier breakdown. Considering most adherent leukocytes were located in venules when examined by retinal perfusion labeling, telmisartan might have suppressed leukocyte adhesion by down-regulating ICAM-1 expression on retinal venules. Although minimal inflammation or no inflammation was observed on day 7 after immunization in vehicle- and telmisartan-treated EAU mice, leukocyte counts in both groups were increased compared with normal mice (4.4 ± 2.1/retina), as we reported previously. Because precise assessment of the very early phase of EAU is difficult, even with histopathologic examination, a retinal perfusion labeling technique was shown to be an ideal method to evaluate the severity of inflammation in the very early phases of EAU.

ARBs are well-established antihypertensive drugs. Telmisartan is an ARB usually used for patients with hypertension and cardiovascular diseases. Among ARBs, telmisartan has the highest affinity for the AT-1 receptor. However, recent studies have revealed various effects of ARBs other than antihypertensive effects, including improvement of insulin resistance, suppression of neovascularization in the eye, and anti-inflammatory effects in several animal models.

In a previous report, losartan, another ARB, was reported to be effective in the suppression of EIU but not effective in EAU. Telmisartan is considered to have stronger antinflammatory effects than other ARBs. In addition, telmisartan is an effective modulator of peroxisome proliferator-activated receptor (PPAR)-γ. The PPAR-γ agonist was originally known as an antidiabetic agent, but it is also a known negative regulator of inflammation and angiogenesis. The suppressive effect of telmisartan on EAU may be mediated partially through the PPAR-γ agonist effect, hence, we are now undertaking experiments to determine whether PPAR-γ signaling is associated with the development of EAU.

Multiple ocular antigens are suggested to be autoimmune in human uveitis, but disease-specific autoantigens are unknown. Even in VKH, a melanocyte-specific autoimmune disease, several autoantigens are reported. Telmisartan ameliorates antigen-nonspecific uveitis EIU and antigen-specific uveitis EAU. Thus, telmisartan is expected to be widely effective in human uveitis irrespective of the involvement of autoimmunity. If telmisartan is also shown to be effective in human uveitis in future studies, it may be a new therapeutic regimen for patients with endogenic uveitis.

References


