

Inhibition of Corneal Neovascularization by Blocking the Angiotensin II Type 1 Receptor

Tomohiko Usui,¹ Kenji Sugisaki,¹ Aya Iriyama,¹ Seiichi Yokoo,¹ Satoru Yamagami,¹ Norihiro Nagai,² Susumu Ishida,² and Shiro Amano¹

PURPOSE. To determine the role of angiotensin II type 1 receptor (AT1R) signaling in corneal neovascularization.

METHODS. Corneal neovascularization was induced by suturing 10-0 nylon 1 mm away from limbal vessels in C57 B/6 mice. Angiotensinogen and its receptor (AT1R) gene expression levels were evaluated by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). The expression of angiotensin II (Ang2) and AT1R was confirmed by Western blotting and immunohistochemistry. To investigate the function of Ang2 in corneal neovascularization, infiltrating macrophages in vascularized corneas and the neovascularized area were investigated after intraperitoneal injection of an AT1R antagonist (telmisartan, 10 mg/kg). Further, corneal mRNA of VEGF, MCP-1, IL-6, ICAM-1, and TNF- α was examined in control and telmisartan-treated mice.

RESULTS. Ang2 and AT1R markedly increased in the neovascularized corneas compared with normal corneas. Ang2 and AT1R were expressed in epithelium and stromal cells (vascular endothelium, infiltrating leukocytes, and keratocytes) in neovascularized cornea at protein levels and were weakly detected in normal corneal epithelium. Infiltrating macrophages were reduced in telmisartan-treated mice on day 7 after suturing. Neovascularized area in the cornea of telmisartan-treated mice was 70% smaller than that of control mice on day 7 after suturing. A PPAR- γ antagonist partially, but significantly, reversed the suppressive effect of telmisartan on induction of corneal neovascularization. The expression of VEGF, MCP-1, IL-6, and ICAM-1 was significantly inhibited in telmisartan-treated mice.

CONCLUSIONS. These findings indicate that Ang2, abundantly expressed in neovascularized corneas, has a significant role in inflammation-related driven corneal neovascularization. AT1R may be a therapeutic target for the suppression of corneal neovascularization. (*Invest Ophthalmol Vis Sci.* 2008;49:4370–4376) DOI:10.1167/iovs.07-0964

From the ¹Department of Ophthalmology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; and the ²Laboratory of Retinal Cell Biology, Department of Ophthalmology, School of Medicine, Keio University, Tokyo, Japan.

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Corresponding author: Tomohiko Usui, Department of Ophthalmology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan 113-8655; tomohiko-uky@umin.ac.jp.

The normal cornea is transparent and maintains itself as an immunoprivileged site, in part because it is avascular. Neovascularization of the cornea often represents a state of disease secondary to a variety of corneal insults, including inflammatory corneal diseases such as corneal infection and chemical injury. Corneal neovascularization is believed to result primarily from an inflammatory disruption of an exquisitely balanced corneal immune system.

Inflammatory reactions in pathologic cornea may stimulate the production of angiogenic factors by local epithelial cells, keratocytes, and infiltrating leukocytes. Some of these factors (i.e., vascular endothelial growth factor [VEGF], monocyte chemoattractant factor 1 [MCP-1], and interleukin 1 [IL-6]) have been identified and isolated from cornea.^{1–4} Angiogenic factors stimulate a localized enzymatic degradation of the basement membrane of perilimbal vessels at the apex of a vascular loop. Vascular endothelial cells migrate and proliferate to form new blood vessels. However, the mechanisms of corneal neovascularization have not yet been fully understood.

The renin-angiotensin system (RAS) is known as the major controller of blood pressure. Angiotensin II (Ang2) is the mediator of this system and the vasoactive peptide generated from inactive angiotensin I by the enzymatic action of angiotensin-converting enzyme (ACE).⁵ Angiotensin I is also generated by the enzyme renin acting on the substrate angiotensinogen.⁵ Ang2 exerts its effects mainly through angiotensin II type 1 receptor (AT1R).⁵ An increasing body of evidence documents the involvement of Ang2 in inflammatory diseases.^{6–9} As an inflammatory mediator, Ang2 enhances vascular permeability through prostaglandins and vascular endothelial growth factor (VEGF)¹⁰ and contributes to the recruitments of inflammatory cells by inducing chemokines and adhesion molecules.^{7,11} Moreover, Ang2 directly induces the proliferation and differentiation of inflammatory cells.⁹ Taken together, studies on the RAS have shed some light on the biology of inflammatory angiogenesis. In ocular tissue, blocking AT1R signaling by AT1R blocker (ARB) suppresses experimental models of choroidal neovascularization¹² and ischemic retinal neovascularization.¹³ These results prompted us to investigate the effect of ARB on the cornea because corneal neovascularization is often associated with inflammatory reactions.^{14–18} In this study, therefore, we investigated the contribution of the RAS and the efficacy of an ARB to inflammatory corneal neovascularization in mice model.

MATERIALS AND METHODS

Animals

All animal experiments were approved by the University of Tokyo Hospital Animal Care Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures were performed with the animals under general anesthesia by xylazine hydrochloride (5 mg/kg) and ketamine hydrochloride (35 mg/kg). C57BL/6 mice (male, 8 weeks; Saitama Experimental Animals, Saitama, Japan) were allowed free access to food and water. A 12-hour day/12-hour night cycle was maintained.

TABLE 1. Primer Pairs

	Sense	Antisense
Angiotensinogen	5'-cac ccc tgc tac agt cca ttg-3'	5'-gtc tgt act gac ccc ctc cag-3'
AT1R	5'-tca cct gca tca tga tct gg-3'	5'-agc tgg taa gaa tga tga tta gg-3'
VEGF	5'-gaa gtc cca tga agt gat cca g-3'	5'-tca ccg cct tgg ctt gtc a-3'
MCP-1	5'-atc cca atg agt agg ctg gag ag-3'	5'-cag aag tgc ttg agg tgg ttg tg-3'
IL-6	5'-ttc ctc tct gca aga gac t-3'	5'-tgt atc tct ctg aag gac t-3'
ICAM-1	5'-gtg tgc agc ttt ggg atg gta-3'	5'-tgt atc tct ctg aag gac t-3'
TNF- α	5'-agc cca cgt cgt agc aaa cca cca a-3'	5'-aca ccc att ccc ttc aca gag caa t-3'
G3PDH	5'-ggt gaa ggt cgg tgt gaa cgg a-3'	5'-tgt tag tgg ggt ctc gct cct g-3'

Induction of Corneal Neovascularization

Corneal neovascularization was induced by nylon suturing under microscopy, according to a previous report.¹⁹ Under general anesthesia, 10-0 nylon suture was placed intrastromally 1 mm away from the limbal vessel. Erythromycin ophthalmic ointment was instilled immediately after surgery.

Semiquantitative RT-PCR

Under a microscope, normal and vascularized corneal buttons in mice were carefully obtained with a 2-mm trephine. Total RNA was isolated from these corneas (Isogen; Nippon Gene, Tokyo, Japan), and cDNA was produced with the use of reverse transcriptase (RT; SuperScript II; Invitrogen, San Diego, CA). No RT samples were used as a negative control. PCR conditions were as follows: appropriate cycles of 45 seconds at 94°C, 45 seconds at 60°C, and 45 seconds at 72°C, with an initial 5-minute denaturation step and a final 7-minute elongation step. The PCR primer pair was selected to discriminate between cDNA and genomic DNA by using primers specific for different exons. Primers are listed on Table 1. Samples were separated in a 2% agarose gel, and the products were visualized with ethidium bromide. An optical scanner was used to determine the densities of the gel bands of the PCR products and to standardize them similarly to those for glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The linear-amplified curve of the PCR product of each sample was examined at four-cycle intervals. Within the linear range of amplification, four sets of PCR products were prepared under appropriate cycling conditions, and the band densities were compared among the groups. NIH Image (version 1.62; National Institutes of Health, Bethesda, MD) was used for the quantification of band density.

Western Blotting

Ang2 and AT1-R protein expression levels in normal and vascularized corneas were evaluated by Western blotting. The eyes were enucleated and the corneal samples were placed in 150 mL lysis buffer (20 mM imidazole HCl, 10 mM KCl, 1 mM MgCl₂, 10 mM EGTA, 1% Triton, 10 mM NaF, 1 mM sodium molybdate, 1 mM EDTA, pH 6.8) supplemented with a protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN) and were sonicated. The lysate was centrifuged at 12,000 rpm for 15 minutes at 4°C. Samples (20 μ g/each lane) were boiled for 5 minutes and separated by SDS-polyacrylamide gel electrophoresis under denaturing conditions and electroblotted to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membranes were incubated in blocking buffer followed by the anti-Ang2 or AT1R polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and then were washed and incubated with a horseradish peroxidase-labeled secondary antibody (Amersham Pharmacia, Piscataway, NJ). The blot was visualized with enhanced chemiluminescence (ECL Plus; Amersham Pharmacia) according to the manufacturer's instructions.

Immunohistochemistry

Eyes were enucleated and embedded in OCT compound, snap frozen in liquid nitrogen, and cut into 7- μ m-thick sections. After fixation with ice-cold acetone, the sections were incubated with anti-Ang2 or AT1R

polyclonal antibody (Santa Cruz Biotechnology) at 2 μ g/mL at room temperature for 1 hour. After washing three times, secondary antibodies (Avidin-Alexa 546 tagged; Molecular Probes, Eugene, OR) were applied for 2 hours at room temperature.

To observe the characterization of positive cells for Ang2 and AT1R, CD45 (leukocyte marker) and CD31 (vascular endothelial cell marker) were chosen for immunohistochemistry. After another three washes, sections were stained with FITC-conjugated rat anti-mouse CD31 (1:50; MEK13.3; BD PharMingen, Cockeysville, MD) or CD45 (1:50; Ly5; BD PharMingen) monoclonal antibody at 4°C overnight. As the negative control for staining, the antibodies were replaced with FITC-conjugated isotype control antibody (IgG2a; BD PharMingen). After washing, sections were viewed under a fluorescein microscope.

Treatment with AT1-R Blocker and PPAR- γ Antagonist

Animals were treated with intraperitoneal injections of an AT1R blocker (telmisartan) or phosphate-buffered saline (PBS) for 1 day before suturing, and the treatment was continued daily until the end of the study with the dose of 5 mg/kg body weight. Telmisartan was a kind gift of Boehringer Ingelheim (Ingelheim, Germany). To evaluate the AT1R blocking and peroxisome proliferator-activated receptor (PPAR)- γ -activating effects of telmisartan, we simultaneously administered telmisartan (5 mg/kg body weight) and a selective PPAR- γ antagonist, GW9662 (Sigma, Tokyo, Japan), at the dose of 1 mg/kg body weight.

Monocyte/Macrophage Counts

Eyes were enucleated 7 days after injury, embedded in OCT compound, snap frozen in liquid nitrogen, and cut into 7- μ m-thick sections. After fixation with ice-cold acetone and blocking with normal goat serum, the sections were stained with rat anti-mouse F4/80 (1:50; macrophage marker; Biomedicals AG, Rheinstrasse, Switzerland) monoclonal antibody to detect infiltrated macrophages and were then stained with FITC-conjugated goat anti-rat IgG2b (1:100; Santa Cruz Biotechnology). The samples were observed under a fluorescence microscope and counted in a masked fashion. Eight serial sections extending through the wound and limbus were studied per cornea. The number of F4/80-positive cells between the limbus and the suture was determined.

Lectin Angiography and Neovascularization Quantitation

Corneal neovascularization was imaged by lectin angiography, as described elsewhere.¹⁹ Mice received intravenous BS-1 lectin conjugated with FITC (Vector Laboratories, Burlingame, CA) and were killed 30 minutes later. The eyes were enucleated and fixed with 1% paraformaldehyde for 15 minutes. After fixation, the corneas were placed on glass slides and studied by fluorescence microscopy (Leica, Deerfield, IL). NIH Image was used for image analysis (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). Neovascularization was quantified by setting a threshold level of fluorescence, above which only

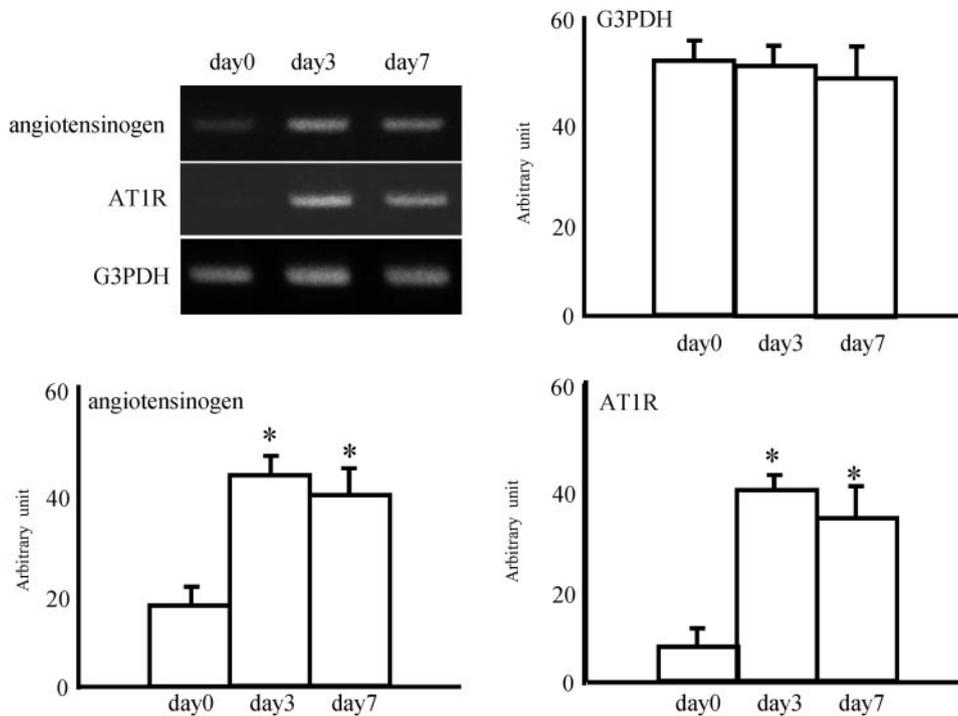


FIGURE 1. Angiotensinogen and *AT1R* gene expression level by semi-quantitative RT-PCR. cDNA, prepared from untreated normal corneas (day 0) and sutured corneas at the indicated points, was subjected to analysis for angiotensinogen and *AT1R* gene expression level. Within the linear range of amplification, four sets of PCR products were prepared, and the band densities were compared. Significantly high angiotensinogen and *AT1R* gene expression levels were detected in postsurgery days 3 and 7 corneal samples compared with those of normal corneas (day 0). * $P < 0.05$.

vessels were depicted. Neovascularization quantitation was performed in a masked manner. The vascularized area was outlined using the innermost vessel of the limbal arcade as the border, and the surface areas of corneal neovascularization (vascularized area/total corneal area) were quantified

Statistical Analysis

The Mann-Whitney *U* test was used to compare the band densities on RT-PCR, neovascularized areas, and F4/80-positive cell numbers. $P < 0.05$ was considered significant.

RESULTS

Angiotensinogen and AT1R Expression in Normal and Vascularized Corneas

RT-PCR was performed to observe the involvement of RAS in corneal neovascularization. Angiotensinogen and *AT1R* gene expression were weakly detected in normal mouse corneas by RT-PCR (Fig. 1) but not in negative control samples (data not shown). Expression levels for those genes increased significantly on days 3 and 7 after surgery compared with expression levels on normal cornea ($P < 0.05$; Fig. 1).

Ang2 and AT1R Expression in Normal and Vascularized Corneas at Protein Levels

To confirm the expression of RAS in corneal neovascularization at protein levels, Western blotting and immunohistochemistry were performed. In Western blotting, the expressions of Ang2 and *AT1R* were also significantly upregulated in vascularized corneas 3 and 7 days after suturing (Fig. 2). Together with Figure 1, these results revealed that the expressions of the RAS system were stimulated in vascularized cornea.

In situ expressions for Ang2 and *AT1R* were investigated by immunohistochemistry. Ang2 and *AT1R* were weakly detected in normal corneal epithelium (Fig. 3). In vascularized corneas, Ang2 was positively stained in corneal epithelium and stromal cells (Fig. 3). Those stromal cells were positively stained by anti-CD45 antibody or anti-CD31 antibody (Fig. 3), with the result that stromal leukocytes and endothelial cells expressed Ang2. *AT1R* was also positively stained in stromal cells (Fig. 3). Those stromal cells were positively stained by anti-CD45 antibody or anti-CD31 antibody (Fig. 3), with the result that stromal leukocytes and endothelial cells expressed *AT1R*.

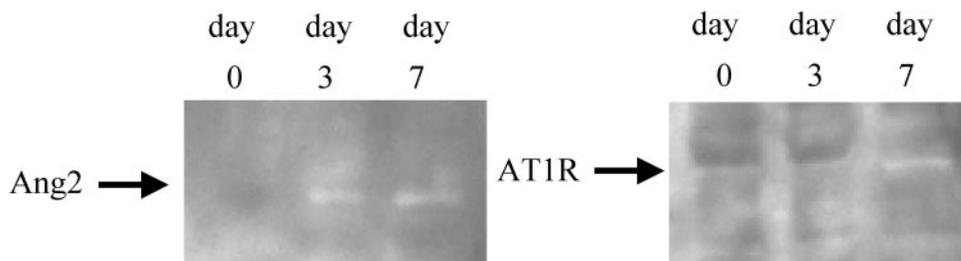


FIGURE 2. Ang2 and *AT1R* protein expression by Western blotting. Proteins prepared from untreated corneas and sutured corneas were subjected to analysis for quantification of Ang2 and *AT1R* at protein level. Significantly high Ang2 and *AT1R* protein expressions were detected in postsurgery days 3 and 7 corneal samples compared with those of normal corneas. Two sets of results on separate experiments were analyzed with similar results. Representative data are shown.

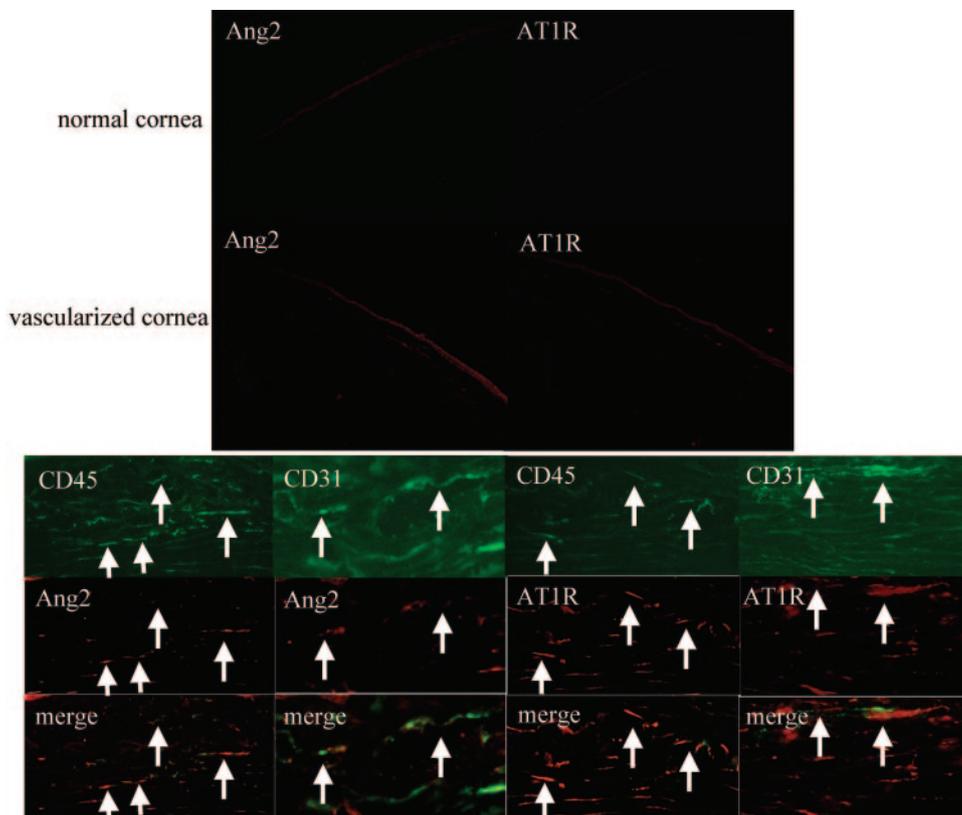


FIGURE 3. Immunohistochemical analysis of Ang2 and AT1R expression in normal and vascularized corneas. Expression of Ang2 and AT1R was determined by immunohistochemistry. Ang2 and AT1R were weakly detected in normal corneal epithelium. In vascularized corneas, Ang2 and AT1R were positively stained in corneal epithelium and stromal cells. These stromal cells were positively stained with anti-CD45 (leukocyte marker) or anti-CD31 (vascular endothelium marker).

Effect of Telmisartan in Corneal Inflammation and Neovascularization

To investigate the role of AT1R signaling in corneal neovascularization, the AT1R blocker telmisartan was administered to mice after suture. We evaluated the number of infiltrated macrophages at the neovascularized areas of corneal stroma. There was statistically significant difference in F4/80-positive cell numbers between telmisartan-treated mice and vehicle-treated mice on day 7 after suturing (Fig. 4; 33.4 ± 5.7 [vehicle] vs. 16.1 ± 2.4 [telmisartan]; $n = 8$ per condition; $P < 0.05$).

Angiogenic responses were also compared between telmisartan-treated mice and vehicle mice (Fig. 5). On day 7 after corneal suture, neovascularized areas in the corneas of telmisartan-treated mice ($n = 8$) were significantly smaller than those in control mice ($n = 8$). Telmisartan-treated mice developed approximately 70% less neovascularization than control mice ($P < 0.01$; Fig. 5C). Therefore, we conclude that AT1R signaling is proangiogenic in corneal neovascularization. It is reported that telmisartan serves as a partial agonist for PPAR- γ .^{20,21} We examined the roles of PPAR to determine whether telmisartan inhibited corneal neovascularization through PPAR- γ signaling. Mice receiving telmisartan at the dose of 5 mg/kg were simultaneously administered a selective PPAR- γ antagonist (GW9662). As shown in Figure 5, telmisartan-induced inhibition of corneal neovascularization was partially reversed by the administration of GW9662 (1 mg/kg). The reversed level was still significantly lower than the level in vehicle-treated mice (Fig. 5). These data showed the inhibitory effects of corneal neovascularization by telmisartan are partially mediated through PPAR- γ signaling.

Effects of Telmisartan on Angiogenic and Inflammatory Molecules

To examine the mechanisms of the inhibitory effect of telmisartan in corneal neovascularization, angiogenic and inflamma-

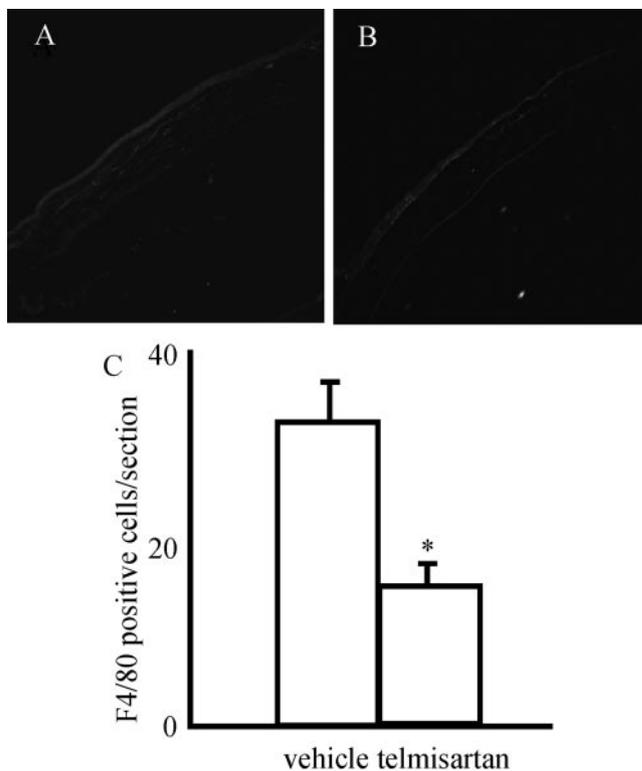


FIGURE 4. Macrophage infiltration in vehicle- and telmisartan-treated mice. Seven days after suturing, the corneas were examined to quantify the number of monocyte lineage cells in the corneal stroma. Immunohistochemical study was performed with antibodies against F4/80, a monocyte/macrophage cell marker. The recruitment of F4/80⁺ cells was suppressed in telmisartan-treated mice (B) compared with vehicle mice (A). Positive cells were counted as described (C). * $P < 0.05$.

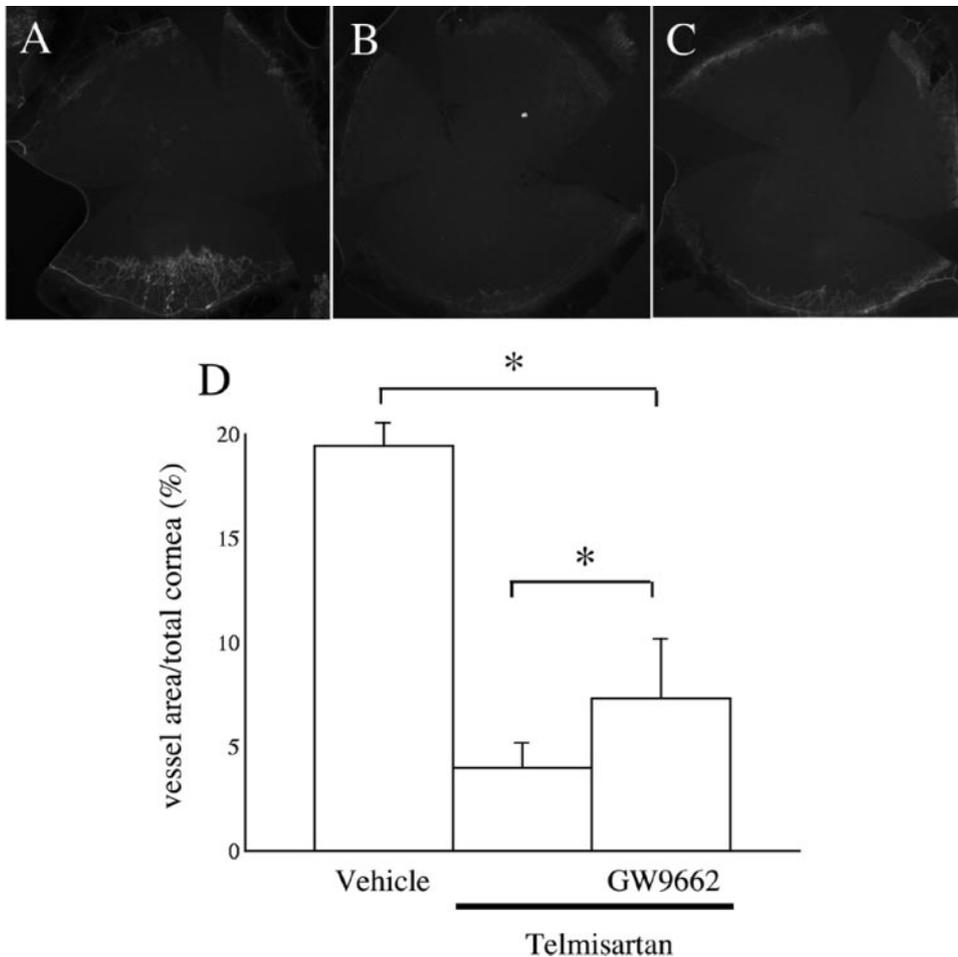


FIGURE 5. Corneal neovascularization in vehicle- and telmisartan-treated mice. Representative angiogenic responses of mouse corneas on day 7 after the intrastromal suturing of 10-0 nylon. Telmisartan-treated mice (B) developed less neovascularization than control mice (A). Selective PPAR- γ antagonist (GW9662) partially but significantly reversed telmisartan-induced suppression of corneal neovascularization (C). Surface areas of corneal neovascularization (vascularized area/total corneal area) were quantified (D). Error bars denote the mean \pm SD. * $P < 0.05$.

tory molecules were analyzed by semiquantitative RT-PCR 7 days after suturing. The systemic administration of telmisartan reduced the mRNA expression of VEGF (both 164 and 120), IL-6, MCP-1, and ICAM-1 (Fig. 6). However, mRNA expression of TNF- α was not inhibited by telmisartan treatment (data not shown).

DISCUSSION

Corneal neovascularization is closely associated with local inflammation, and many inflammatory mediators are involved in this process.^{14–18} In our findings, RAS expression was upregulated in the vascularized cornea compared with the normal cornea. Corneal inflammation and neovascularization were significantly reduced in AT1R blocker in telmisartan-treated mice compared with control mice. These findings imply that stimulation by suturing increases Ang2 and AT1R expression in the cornea and that AT1R signaling has a critical role in inflammatory corneal neovascularization.

Based on the results of the present study, Ang2 expression levels are low under normal conditions on the ocular surface. This may be reasonable for corneal immune privilege. However, when stimulation occurs, activated corneal epithelia and stromal cells might become the major sources of Ang2 expression. Overexpression of Ang2 in local cornea may then exert and accelerate various inflammatory responses. In fact, a local angiotensin-generating system in addition to the circulating RAS is present in several tissues. The so-called tissue RAS can act locally as a paracrine or an autocrine factor in meeting specific needs for individual tissues, and it can operate, in

whole or in part, independently of the circulating counterpart.²² Because cornea is avascular tissue and is maintained by vessel-independent metabolism, it may be surprising that avascular cornea is also involved in the RAS. However, a study by Savaskan et al.²³ showed that the expression of RAS components, among them Ang2, angiotensin-converting enzyme, and AT1R in human cornea and conjunctiva. AT1R signaling is also involved in conjunctival wound healing.²⁴ In the other avascular tissue, synovium expressed the RAS component in rheumatoid arthritis (RA), and an ARB suppressed the inflammatory reaction of RA in an animal model.²⁵ Taken together, in pathologic conditions, corneal RAS is activated and accelerates proangiogenic and proinflammatory activities. To our knowledge, this is the first report demonstrating that corneal RAS exists and is involved in corneal abnormalities, including corneal neovascularization *in vivo*.

To observe the effect of ARB in corneal neovascularization, we injected telmisartan intraperitoneally and examined corneal inflammation, neovascularization, and the expression of inflammatory molecules (Figs. 4–6). Given that corneal neovascularization is usually associated with inflammatory disorders, inflammatory cells, including macrophages, are recruited in vascularized corneas. The infiltrating macrophages are thought to be essential to induce corneal neovascularization in a similar model.²⁶ Although the recruitment of leukocytes to inflammatory sites is mediated by growth factors and chemokines, AngII also functions as a proinflammatory mediator. ARB treatment hampered endothelial cell migration and proliferation by blocking growth factors such as VEGF and macrophage infiltration in corneal neovascularization.

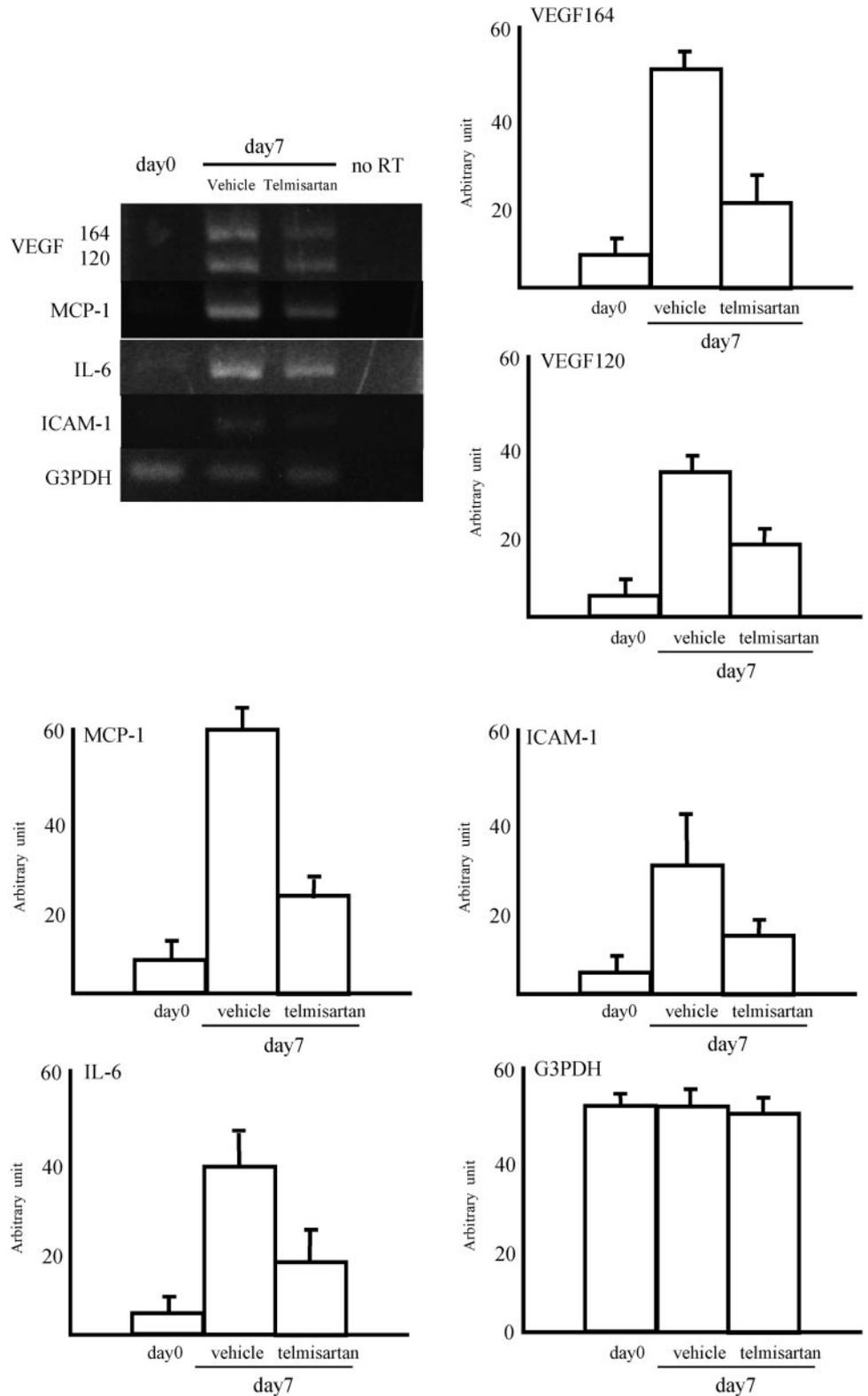


FIGURE 6. Effects of AT1R blockade on corneal expression of angiogenic and inflammatory molecules analyzed by RT-PCR. Within the linear range of amplification, PCR products were prepared, and the band densities were compared. AT1R blockade by telmisartan significantly suppressed corneal VEGF164, VEGF120, MCP-1, IL-6, and ICAM-1. Four sets of results on separate experiments were analyzed with similar results. Representative data are shown. * $P < 0.05$.

ARB treatment resulted in the suppression of VEGF, ICAM-1, MCP-1, and IL-6, which were upregulated after the induction of corneal neovascularization (Fig. 6). These results are compatible with findings in a choroidal neovascularization model of mice.¹² The inhibition of those molecules by ARB may result in the suppression of inflammation and of the angiogenic response in cornea. Among those inflammatory molecules, VEGF

is one of the potent stimuli in corneal neovascularization^{1,27} and is involved in various angiogenic processes. VEGF promotes endothelial migration, proliferation, and monocyte migration to accelerate vascular permeability and angiogenesis.²⁷ VEGF also upregulates adhesion molecules such as ICAM-1 through VEGFR2.²⁷ It has been reported that Ang2 induces *in vitro* expression of VEGFR2 mRNA.²⁸ Thus, ARB treatment can

efficiently reduce VEGF signaling. In addition to VEGF, other potent angiogenic factors—MCP-1 and IL-6—were also ameliorated by ARB treatment (Fig. 6). Furthermore, specific pharmacologic function in telmisartan, unlike other ARB, is a PPAR- γ partial agonist.^{20,21} Because PPAR- γ ligand works in an anti-inflammatory role,^{20,21} telmisartan may have advantage for reducing inflammation. In fact, one of the PPAR- γ agonists, pioglitazone, inhibited corneal neovascularization in a rat model.²⁹ Therefore, dramatic inhibition of corneal neovascularization by ARB might be observed in the present study (Fig. 5).

In conclusion, our data define the biological significance of the RAS even in avascular cornea. Corneal neovascularization is one of a major sight-threatening conditions and can lead to corneal scarring, edema, lipid deposition, and inflammation that may not only diminish visual acuity but may also decrease the success rate of subsequent penetrating keratoplasty.³⁰ Therefore, suppressing and inhibiting corneal neovascularization is important to maintain ocular homeostasis and to protect sight. AT1R blockade may be a potent strategy to inhibit inflammatory corneal neovascularization, and topical administration of ARB may be able to prevent neovascularization in cornea.

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