Role of IL-6 in Angiotensin II-Induced Retinal Vascular Inflammation

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PURPOSE. The production of proinflammatory cytokines has been shown to play a critical role in a variety of retinal vascular diseases. Angiotensin II and VEGF have been implicated in the initiation of vascular inflammation and retinal vascular disease. However, detailed mechanisms of this process and interactions between inflammatory agonists and angiotensin II in promoting retinopathy are poorly understood. The present study was an investigation of the role of interleukin (IL)-6 in angiotensin II-induced retinopathy.

METHODS. Rats and IL-6-deficient and wild-type mice were treated with angiotensin II or IL-6, and their retinas were analyzed for leukocyte adhesion or for the expression and localization of VEGF or IL-6. Leukocyte adhesion was assayed by concanavalin A labeling. Vascular density was determined by morphometric analysis. NADPH oxidase activity was assayed by dihydroethidium imaging of superoxide.

RESULTS. Intravitreal injection of angiotensin II caused increases in IL-6 mRNA and protein and in leukocyte adhesion to the retinal vessels. IL-6 protein was localized to CD11b-positive microglia and macrophage-like cells. Angiotensin II treatment stimulated increases in retinal levels of VEGF expression and NADPH oxidase activity, which were associated with increased surface area and remodeling of the retinal vessels. These effects were blocked by knocking out IL-6. Intravitreal IL-6 directly induced leukocyte adhesion in both wild-type and IL-6-deficient mice.

CONCLUSIONS. The results indicate that IL-6 expression is essential for angiotensin II-induced increases in retinal VEGF expression, leukostasis, and vascular remodeling. The data suggest a critical role for IL-6 in mediating angiotensin II-induced retinal

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Retinal vascular inflammation is a common feature of blindness diseases such as diabetic retinopathy, retinopathy of prematurity (ROP), and choroidal neovascularization.¹ Interleukin (IL)-6 is a potent proinflammatory cytokine involved in many pathologic processes characterized by vascular inflammation and injury, including proliferative diabetic retinopathy, choroidal neovascularization, atherosclerosis, and cancer.^{2–7}

Vascular inflammation is a complex process that is thought to be initiated by activation of the immune system leading to increased expression of the angiogenesis/permeability factors vascular endothelial growth factor (VEGF), leukocyte attachment protein intercellular adhesion molecule (ICAM)-1, and monocyte chemoattractant protein-1 (CCL-2).^{1,8} Previous studies have shown that IL-6 shares common characteristics with VEGF, in that both are induced by hypoxia and hyperglycemia and both play a role in vascular inflammation, vascular permeability, and pathologic angiogenesis.^{9–16} IL-6 has been shown to induce VEGF expression in models of choroidal neovascularization and tumor angiogenesis.^{4,7} However, the specific role of IL-6 in retinal vascular disease is unclear.

Accumulating evidence has indicated that angiotensin II, the effector protein of the renin angiotensin system (RAS) has a fundamental role in the pathogenesis of retinal vascular diseases, including retinopathy of prematurity, proliferative diabetic retinopathy, choroidal neovascularization, and endotoxin-induced uveitis.^{17–22} In addition to its well-known vasoconstriction activity, angiotensin II is a potent inducer of vascular growth and inflammation. Other studies have demonstrated that intravitreal delivery of angiotensin II in rats induces VEGF expression and vascular inflammation, as shown by increased leukocyte adhesion to the retinal vessels in a process that requires reactive oxygen species (ROS) generation.¹⁸ Angiotensin II can also induce increases in VEGF expression in vitro by increasing ROS formation.²³ The proinflammatory action of angiotensin II has been associated with increased expression of IL-6 in models of peripheral vascular disease. This process is thought to play a critical role in the development of vascular inflammation.^{6,24,25} The specific role of IL-6 in vascular injury is not yet understood, but upregulation of VEGF is thought to be involved.^{4,7} The purpose of this study was to investigate the mechanism by which angiotensin II induces retinal vascular inflammation and determines the specific role of IL-6 in this process.

MATERIALS AND METHODS

Animal Models

All procedures with animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision

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Research and were approved by the institutional animal care and use committee (Animal Welfare Assurance no. A3307-01).

Sprague-Dawley rats were injected intravitreally with angiotensin II (20 $\mu\text{g}/5 \mu\text{L}$) or saline (5 μL) as described by Chen et al.¹⁸ After 24 hours, the rats were killed and the eyes enucleated. One eyeball from each rat was embedded in OCT and frozen in liquid nitrogen for cryosectioning. The contralateral retina was dissected, frozen in liquid nitrogen, and used for measurement of IL-6 mRNA by quantitative RT-PCR.

Wild-type C57BL6 and IL-6-deficient mice (Jackson Laboratories, Bar Harbor, ME) were treated with angiotensin II injected intravitreally (4 $\mu\text{g}/1 \mu\text{L}$, 48 hours). This dosage was selected based on the assumption that the vitreous volume of the mouse is approximately one fifth that of the rat. Another group of mice was treated by subcutaneous infusion of angiotensin II with minipumps (5 mg/kg/d for 2 weeks; Alzet, Cupertino, CA). Age-matched wild-type and IL-6-knockout mice served as the control. Retinas from the mice that received intravitreal angiotensin II injections were prepared for analysis of IL-6 protein by ELISA and leukostasis by labeling adherent leukocytes with concanavalin A, as described previously.²⁶ The retinas from the chronically treated mice were processed for frozen sectioning and immunolocalization or Western blot analysis. To study the direct effect of IL-6, we injected wild-type C57BL6 and IL-6-deficient mice intravitreally with IL-6 (10 ng/1 μL , 24 hours) and analyzed them for leukostasis. The dosage was based on our preliminary study in wild-type mice, which found that intravitreal injections of IL-6 induce moderate leukostasis at 1 ng/eye and a prominent increase at 10 and 100 ng per eye.

RT-PCR Assay

Total RNA was isolated (RNA 4PCR kit; Applied Biosystems, Inc. [ABI] Foster City, CA) according to the manufacturer's instructions. Total RNA was reverse transcribed with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) to generate cDNA, and the relative amounts of IL-6 and 18S transcript were determined by real-time quantitative PCR with gene-specific primers (*Taqman* assay; ABI). The level of 18S was used as the internal control and IL-6 in the vehicle-treated group was set as the reference. MCP-1 gene expression was determined by real-time quantitative PCR in PCR master mix (Power SYBR green; ABI) with the following primers: rat CCL2, forward, 5'-CTC AGC CAG ATG CAG TTA ATG C-3'; and rat CCL2, reverse, 5'-AGC CGA CTC ATT GGG ATC AT-3'.

IL-6 ELISA

Retina lysates were prepared as described elsewhere.²⁷ IL-6 protein levels were determined in the lysate (200 μg in 50 μL ; Bio-Plex Pro Mouse Cytokine IL-6 Set and a Bio-Plex reader; Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Western Blot Analysis

Retinas were homogenized in a modified RIPA buffer (20 mM Tris-HCl; pH 7.4), 2.5 mM ethylenediamine tetraacetic acid, 50 mM NaF, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, and 1 mM phenylmethyl sulfonyl fluoride). Protein samples were processed according to the method described by Ferrara and Henzel.²⁸ Briefly, protein samples (100 μg) were adjusted to a volume of 1 mL with 10 μM Tris (pH 7.4) and 100 μM NaCl and incubated overnight with 50 mL of equilibrated heparin-agarose beads (Sigma-Aldrich, St. Louis, MO). The samples were boiled in sodium dodecyl sulfate sample buffer (100°C, 10 minutes), to elute the proteins, which were then electrophoresed in 4% to 20% Tris-HCl gradient gels (Bio-Rad Laboratories), transferred to nitrocellulose membranes, and probed with anti-VEGF antibody (Oncogene, San Diego, CA).

Leukostasis

Retinal leukostasis was assayed by labeling the adherent leukocytes with concanavalin A (Vector Laboratories, Burlingame, CA), according to a published method.²⁶

Immunolocalization

Frozen sections (10 μm) were fixed in 4% paraformaldehyde for 30 minutes, washed with PBS (pH 7.5), and permeabilized with Triton X (0.3% in PBS with normal goat serum 5%). IL-6 was detected by using a biotinylated anti-IL-6 antibody (BD Biosciences, San Jose, CA) at a dilution of 1:200 (2 hours) followed by avidin-FITC (1:300, in HEPES [pH 8.2], 30 minutes; Vector Laboratories). VEGF was detected with rabbit anti-VEGF (1:400; Abcam, Cambridge, MA), followed by reaction with a goat anti-rabbit antibody (1:500; Invitrogen). CD11b was detected with mouse anti-CD11b/c (Biolegend, San Diego, CA) followed by goat anti-mouse antibody (Invitrogen).

For immunolabeling of retinal vessels, frozen sections (10 μm) were fixed with paraformaldehyde (4%, 5 minutes) followed by blocking of endogenous biotin with a kit from Sigma-Aldrich and then incubated 24 hours with *Griffonia simplicifolia* isolectin B4 (GSI; Vector Laboratories) at 1:50 (in PBS with 0.3% Triton X-100 and 5% normal goat serum). The sections were washed in PBS, labeled with Texas red-conjugated avidin D (1:120, 1 hour at room temperature; Vector Laboratories), and covered with antifade medium (Vector Laboratories), and images were collected with a microscope (Axiovision; Carl Zeiss Meditec, Inc., Dublin, CA). Vascular density was determined by counting the lectin-positive vascular profiles/retina (Metamorph Software; Molecular Devices, Downingtown, PA). The counts were performed by two blinded observers. Some frozen sections from each group were stained with hematoxylin and eosin (H&E) according to the Fisher Scientific protocol for Harris hematoxylin staining.

Dihydroethidium Assay for Superoxide Formation

To evaluate production of superoxide in situ, we used the oxidative fluorescent dye dihydroethidium (DHE). DHE freely permeates cells and in the presence of $\text{O}_2^{\cdot -}$ is oxidized to ethidium bromide, which binds to DNA and fluoresces red. Frozen sections were preincubated in NADPH (100 μM), NADPH with PEG-SOD (400 U/mL), or NADPH with apocynin (1 mM) for 20 minutes followed by DHE (2 μM ; 20 minutes, 37°C). Apocynin (4-hydroxy-3-methoxy-acetophenone) specifically blocks activity of NADPH oxidase by preventing assembly of the cytosolic NADPH oxidase components (p40phox, p47phox, and p67phox) with the membranous components gp91phox and p22phox.²⁹ DHE images from serial sections treated with or without inhibitors were obtained with a fluorescence microscope (Axiovision; Carl Zeiss Meditec, Inc.). DHE is excited at 488 nm with an emission spectrum of 610 nm. The images were analyzed for reaction intensity (Metamorph Image System; Molecular Devices).

Statistical Analysis

The results are expressed as the mean $\pm$ SEM. Group differences were evaluated by one-way ANOVA followed by post hoc analysis. The results were considered significant at $P < 0.05$.

RESULTS

Upregulation of IL-6 by Angiotensin II

Studies in rats have shown that intravitreal injections of angiotensin II (20 $\mu\text{g}/\text{eye}$) cause increases in retinal levels of VEGF expression and leukocyte adhesion to the walls of the retinal vasculature, suggesting a mechanism involving production of inflammatory mediators.¹⁸ To see whether upregulation of IL-6 could be involved in this process, we determined the effects of intravitreal injections of angiotensin II (20 $\mu\text{g}/5 \mu\text{L}$, 24 hours) on IL-6 expression in the rat retina. Immunolocalization studies showed that numerous large cells in the inner retina were intensely reactive for IL-6 in the angiotensin II-injected animals. IL-6 immunoreactivity was also observed in a population of small, microglia-like cells with stellate processes in both the inner and outer retina. By contrast, in the retinas from the

FIGURE 1. Upregulation of IL-6 expression by angiotensin II. **(A)** Confocal imaging of retinal sections double labeled with anti-IL-6 (green) and anti-CD11b (red). In the retinas of control rats injected intravitreally with vehicle (Veh, saline, 5 μ L), IL-6 was localized to CD11b-positive, microglia-like cells (arrows) in the outer retina. In the retinas of rats injected intravitreally with angiotensin II (AngII, 20 μ g/5 μ L), IL-6 was localized to large CD11b-positive cells (arrowheads) in the inner retina as well as to processes of CD11b-positive microglia-like cells (arrows) in both the inner and outer retina. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Magnification, $\times 200$. **(B)** Quantitative real-time RT-PCR analysis of IL-6 and MCP-1 mRNA. IL-6 and MCP-1 mRNA significantly increased in retinas of rats treated with angiotensin II compared with the levels in the vehicle control ($n = 6$, $*P < 0.05$). **(C)** Analysis of IL-6 protein by ELISA. IL-6 protein was prominently increased in retinas of wild-type mice treated with angiotensin II, whereas it was below the detection sensitivity (not detectable, N.D.) in the vehicle control or IL-6-deficient (IL-6ko) mice treated with angiotensin II.

vehicle control animals, IL-6 expression was restricted to small, microglia-like cells in the outer nuclear layer (Fig. 1A). The other retinal layers were negative for IL-6. Double-labeling studies with the microglial/macrophage marker CD11b showed that both the small and the large IL-6-positive cells were also positive for CD11b (Fig. 1B), suggesting that the IL-6-positive cells were microglia and macrophages. Quantitative real-time RT-PCR analysis of the contralateral retinas from the same animals used for immunolocalization studies showed that intravitreal injection of angiotensin II resulted in a ~ 9 -fold increase in IL-6 mRNA and a ~ 12 -fold increase in MCP-1 mRNA compared with the saline-injected controls (Fig. 1B). IL-6 protein was also determined in angiotensin II-treated mice. The level of IL-6 in both the vehicle-treated wild-type mice and angiotensin II-treated IL-6ko mice was very low and under the detection limit of the kit (1.5 pg/mL). However, the average

level of IL-6 in the retina lysates from the angiotensin II-treated wild-type mice was markedly increased (3.1 pg/mL, Fig. 1C).

Suppression of Angiotensin II-Induced Inflammation in Mice Deficient in IL-6

To determine the specific contribution of the angiotensin II-induced increases in IL-6 in the retinal vascular inflammatory reaction, we determined the effects of intravitreal injections of angiotensin II (4 μ g/1 μ L, 48 hours) on retinal leukostasis and vascular morphology in the wild-type and IL-6-deficient mice. Leukostasis was assayed by using concanavalin A to label the adherent leukocytes. The results confirmed that intravitreal injections of angiotensin II caused substantial increases in leukocyte attachment to the vessel walls (Fig. 2). This effect was completely blocked in the IL-6-deficient mice ($P < 0.05$, $n = 3$).

A

B

FIGURE 2. Suppression of angiotensin II-induced increases in retinal vascular leukostasis in IL-6-deficient mice. **(A)** Flatmount images of concanavalin A-labeled retinas showing adherent leukocytes (*arrows*) within the retinal vessels of wild-type (WT) and IL-6-deficient (IL-6ko) mice injected intravitreally with vehicle (saline, 1 μ L) or angiotensin II (AngII, 4 μ g/1 μ L). **(B)** The results of a quantitative analysis of the number of adherent leukocytes/retina. $n = 3$, $^{\#}P < 0.05$ versus vehicle control (Veh). $^{*}P < 0.05$ versus AngII-treated WT.

We then determined the effects of angiotensin II treatment on retinal vascular morphology. For these experiments, we used wild-type and IL-6-deficient mice implanted with osmotic minipumps that delivered angiotensin II for 2 weeks (5 mg/kg/d). Imaging of the retinal vessels by GSI B4 immunostaining (Fig. 3A) showed that angiotensin II treatment of the wild-type mice caused significant disruption of the normal vascular patterning. As shown in Figure 3A, the retinal vessels appeared larger and more densely packed in the angiotensin II-treated wild-type mice than in the untreated control animals. By contrast, distribution and morphology of the vessels in the retinas of the angiotensin II-treated IL-6-deficient mice appeared similar to that in the untreated wild-type or IL-6-deficient control animals. Double-blind morphometric analysis of retinal vascular density confirmed a 16% increase in vascular surface area in the angiotensin II-treated wild-type mice compared with the untreated wild-type controls, whereas vessel density in the angiotensin II-treated IL-6-deficient mice was reduced by 29% compared with that in the treated wild-type mice ($P < 0.05$, Table 1).

Examination of retinal structure in adjacent H&E-stained sections prepared from the same mice showed striking abnormalities in retinal structure in the angiotensin II-treated wild-type mice. The angiotensin II-treated wild-type retinas had apparent disruptions of the inner limiting membrane and nerve fiber layer, and there were numerous large vacuoles within the subretinal space and inner nuclear and outer plexiform layers (Fig. 3B). By contrast, the retinas of the IL-6-deficient mice treated with angiotensin II had a few small vacuoles in the ganglion cell layer and inner and outer nuclear layers, but were otherwise similar to the untreated controls. The retinas of the IL-6-deficient mice were structurally normal.

Suppression of Angiotensin II-Induced Increases in VEGF in Mice Deficient in IL-6

To assess the role of IL-6 in mediating the proinflammatory actions of angiotensin II, we next compared the effects of angiotensin II treatment on VEGF expression in retinas from wild type and IL-6-deficient mice. The results of these experiments confirmed the previous reports that angiotensin II treatment increases retinal VEGF expression.¹⁸ Western blot analysis showed that levels of VEGF protein were increased by 2.3-fold in the retinas of angiotensin II-treated wild-type mice compared with the untreated control animals (Fig. 4A). This effect was completely absent in the IL-6-deficient mice ($P < 0.05$, $n = 3$). VEGF levels in the vehicle-treated IL-6-deficient mice were comparable with that in the wild-type control mice. These results indicate that IL-6 is necessary for angiotensin II-induced upregulation of VEGF expression in this model.

Immunolocalization studies of frozen sections from contralateral retinas of the same mice showed prominent increases in VEGF immunoreactivity in the retinas of the angiotensin II-treated mice. Double labeling with GSI B4 and anti-VEGF antibody showed that VEGF was mainly localized to the retinal vessels. Immunostaining of retinal sections from angiotensin II-treated, IL-6-deficient mice showed a pattern of VEGF-positive retinal vessels similar to that in the untreated control retinas (Fig. 4B).

Suppression of Angiotensin II-Induced Activation of NADPH Oxidase in Mice Deficient in IL-6

To test whether activation of NADPH oxidase could play a role in the angiotensin II-induced alterations seen in this experi-

A **B**

FIGURE 3. Suppression of angiotensin II-induced vascular remodeling/hypertrophy in IL-6-deficient mice. **(A)** Fluorescence microscope images of GSI-labeled retinal vessels in wild-type (WT) or IL-6-deficient (IL-6ko) mice treated for 2 weeks with vehicle (Veh) or angiotensin II (AngII, 5 mg/kg/d, SC). Vessels appeared larger and more densely packed in the angiotensin II-treated wild-type mice than in the angiotensin II-treated IL-6-deficient mice. **(B)** Light microscope images of H&E-stained retinal sections from the vehicle and angiotensin II-treated wild-type and IL-6-deficient mice. The retinal lamination pattern was distorted by large vacuoles in the angiotensin II-treated wild-type mice.

mental model, we assayed superoxide production in the same retinal specimens with DHE imaging. DHE reacts with superoxide to produce a red fluorescent signal that can be detected by confocal imaging. The results showed that flash-frozen sections from angiotensin II-treated wild-type mice were strongly positive for the superoxide reaction (Fig. 5). The signal was completely blocked by pretreatment with the specific NADPH

inhibitor apocynin (1 mM) or with superoxide dismutase (SOD; 400 U/mL), indicating the specificity of the reaction for NADPH oxidase and superoxide anion, respectively. This angiotensin II-induced increase in the DHE reaction was not seen in the IL-6-deficient mice. Quantification of the fluorescent signal with computer-assisted densitometry showed a statistically significant difference between the angiotensin II-treated wild-type mice and the untreated controls or IL-6-deficient mice ($P < 0.05$, $n = 3$).

TABLE 1. Morphometric Analysis of Retinal Vascular Density

	Wild-Type	Wild-Type/AngII	IL-6ko	IL-6ko/AngII
Mean	229.3	266.3*	218.5	188.5†
SEM	3.6	5.8	11.6	3.5

Wild-type or IL-6-deficient (IL-6ko) mice were treated with vehicle or angiotensin II (AngII; 90 ng/min, 2 weeks).

* $P < 0.05$ versus wild-type.

† $P < 0.05$ versus wild-type/AngII ($n = 4$).

Induction of Retinal Inflammation by IL-6

To access whether IL-6 is sufficient to induce retinal inflammatory response, we determined the effects of intravitreal injections of IL-6 (10 ng/1 μ L, 24 hours) on retinal leukostasis in the wild-type and IL-6-deficient mice. The results showed that intravitreal injections of IL-6 caused substantial increases in leukocyte attachment to the vessel walls compared with that in the vehicle control (Fig. 6, $P < 0.05$). There was no difference

A **B**

FIGURE 4. Suppression of angiotensin II-induced upregulation of VEGF expression in IL-6-deficient mice. **(A)** Western blot analysis of relative optical density of VEGF protein in retinal extracts from wild-type (WT) and IL-6-deficient (IL-6ko) mice treated with vehicle (Veh) or angiotensin II (AngII, 5 mg/kg/d, SC, 2 weeks). $n = 3$, # $P < 0.05$ versus Veh; * $P < 0.05$ versus AngII-treated WT. *Top*: a representative Western blot. **(B)** Fluorescence microscope images of retinal sections labeled with GSI B4 (red) and anti-VEGF (green) in wild-type and IL-6-deficient (IL-6ko) mice treated with angiotensin II (AngII) or vehicle (Veh). VEGF colocalizes with the GSI-positive retinal vessels in all groups and is markedly increased in the hypertrophic vessels of the angiotensin II-treated wild-type mice.

in leukostasis between the wild-type and IL-6-deficient mice, suggesting that both genotypes respond to IL-6 similarly.

DISCUSSION

The goal of this study was to evaluate the role of IL-6 in angiotensin II-induced retinal vascular inflammation. In our study, angiotensin II increased IL-6 expression in the retina and deletion of IL-6 blocked angiotensin II-induced increases in leukocyte adhesion to the vessel wall. Angiotensin II caused structural remodeling and increases in the surface area of the retinal vessels. Our data further indicate that the angiotensin II-induced vascular alterations involved activation of NADPH oxidase and increases in VEGF expression and that deletion of IL-6 prevented all these effects.

The action of angiotensin II in increasing the expression of IL-6 in macrophages and peripheral vascular cells and tissues has been demonstrated by studies in cultured cells and animal models.^{6,25,30} However, to our knowledge, our study is the first to show that angiotensin II increases IL-6 expression within the retina. Our data showed that intravitreal injection of angiotensin II caused increases in levels of IL-6 mRNA and protein. Immunolocalization study showed that IL-6 was localized to CD11b-positive retinal microglia and/or macrophages. Many of the double-positive cells in the inner retina had large cell bodies, suggesting an activated phenotype. The intensity of the IL-6 immunoreactivity in microglia-like cells within the outer nuclear layer was similar in both the angiotensin II and saline-treated control retinas, suggesting that IL-6 is normally expressed in retinal microglia. Studies in a mouse model for glaucoma have shown that increases in microglial cell expres-

A

FIGURE 5. Suppression of angiotensin II-induced activation of NADPH oxidase in IL-6-deficient mice. **(A)** Real-time DHE imaging of superoxide formation in retinas from wild-type (WT) and IL-6-deficient (IL-6ko) mice treated with or without angiotensin II (Ang II, 5 mg/kg/d, 2 weeks). Angiotensin II treatment increased the DHE reaction in the wild-type retinas. This effect was blocked in the IL-6-deficient mice and when sections were pretreated with SOD (400 U/mL) or apocynin (1 mM). **(B)** Statistical analysis of fluorescence intensity indicates a significant increase in superoxide formation in wild-type mice treated with angiotensin II. Deletion of IL-6 blocked this effect. The fluorescence reaction was almost completely eliminated by pretreatment with SOD or apocynin. $n = 3$, # $P < 0.05$ versus WT/Veh, * $P < 0.05$ versus WT/AngII, ^ $P < 0.05$ versus untreated.

B

sion of IL-6 is associated with increases in intraocular pressure, whereas microglial cells in retinas with low intraocular pressure express low levels of IL-6.³¹ In our study, the IL-6 increase in the inner retina was clearly due to the angiotensin II treatment, because the inner retinal layers of the saline-treated control animals were negative for IL-6.

An association between elevated levels of IL-6 and retinopathy has been suggested previously based on data showing increases in IL-6 expression in samples from patients and animal models of retinal vascular disease. For example, analyses of vitreous samples from patients with diabetic retinopathy have shown increases in IL-6 protein levels.^{5,9,12} Moreover, diabetes-induced increases in vitreous levels of IL-6 have been shown to correlate with increases in VEGF and with the severity of diabetic retinopathy, suggesting a relationship between IL-6 and disease progression.^{9,12,32} Increased levels of IL-6 correlated with VEGF increases in the aqueous humor and vitreous of patients with macular edema, suggesting a role for IL-6 in hyperpermeability.^{9,14,33} Increases in IL-6 expression have also been reported in retinas of rats with diabetes and ischemia-reperfusion injury and mice with choroidal neovascularization.^{4,34}

IL-6 has been shown to be involved in angiotensin II-induced endothelial dysfunction and hypertrophy.²⁵ Its specific role in retinopathy is not yet clear. However, studies in a model of laser-induced choroidal neovascularization have shown that blockade of IL-6 signaling or deletion of IL-6 inhibits subretinal neovascularization, implying IL-6 involvement in pathologic angiogenesis.⁴ Our finding that the angiotensin II-induced increase in retinal leukostasis was prevented in IL-6-deficient mice indicates that IL-6 also has a key role in angiotensin II-induced retinal vascular injury. This notion was further supported by our finding that IL-6 directly induces leukostasis in both wild-type and IL-6-deficient mice. Moreover, the angiotensin II-induced retinopathy was associated with structural remodeling of the retinal vessels as indicated by their abnormal patterning and increased surface area. Each of these alterations was prevented in the IL-6-deficient mice, suggesting a cause-and-effect relationship. These results are consistent with the previous finding that upregulation of IL-6 is involved in angiotensin II-induced dysfunction and hypertrophy of peripheral vessels.²⁵

To our knowledge, our study is the first to show that angiotensin II treatment causes structural remodeling of the

A

B

FIGURE 6. IL-6 induced leukostasis in both wild-type and IL-6-deficient mice. **(A)** Flatmount images of concanavalin A-labeled retinas showing adherent leukocytes (arrows) within the retinal vessels of wild-type (WT) and IL-6-deficient (IL-6ko) mice injected intravitreally with vehicle (saline, 1 μ L) or IL-6 (10 ng/1 μ L). *Insets:* a high-magnification view of the attached leukocytes in the IL-6-treated retinas. Magnification, $\times 400$. **(B)** Results of quantitative analysis of the number of adherent leukocytes/retina. $n = 4$, * $P < 0.05$ versus WT/Veh.

retinal vessels. However, retinal vascular remodeling and endothelial hyperplasia and hypertrophy have been described in the retinas of monkeys treated with intravitreal injections of VEGF.^{35,36} Additional studies in which electron microscopy techniques are used are needed to see whether endothelial cell hyperplasia and hypertrophy also occur in retinas of angiotensin II-treated mice. However, our finding that increases in VEGF protein correlated with the alterations in vascular patterning and increased surface area suggests that a mechanism of VEGF-induced vascular remodeling may also be operative in this model.

The mechanisms of angiotensin II-induced retinal vascular inflammation and remodeling/hypertrophy are not yet clear, but appear to involve activation of NADPH oxidase and VEGF expression. Activation of NADPH and ROS formation is known to have a role in the upregulation of VEGF expression and retinal inflammation during retinopathy.^{10,26,37} Angiotensin II-induced increases in leukostasis have been shown to be associated with increases in NADPH oxidase-dependent ROS formation and VEGF expression and to be prevented by treatment with specific inhibitors of NADPH oxidase or VEGF.¹⁸ In our studies of DHE imaging and Western blot techniques, angiotensin II induced increases in NADPH oxidase activity and VEGF protein expression, respectively. Furthermore, both of these alterations were significantly reduced in the IL-6-deficient mice, suggesting that the angiotensin II-induced increase in IL-6 expression is responsible. The potential involvement of IL-6 in VEGF overexpression during vascular disease has been suggested by studies showing that laser-induced choroidal neovascularization is reduced in mice lacking IL-6.⁴ However, to our knowledge, our data are the first to show the involvement of IL-6 in angiotensin II-induced activation of NADPH oxidase and VEGF overexpression in the retina.

In summary, angiotensin II is a peptide with a wide range of functions, including the regulation of blood pressure, cellular differentiation and growth, induction of inflammation and recruitment of inflammatory cells, including macrophages and microglial cells, which can secrete proangiogenic growth factors and cytokines.²⁴ Our data indicate that angiotensin II induces retinal vascular inflammation when administered acutely and that chronic exposure causes vascular remodeling and hypertrophy, via a mechanism involving expression of IL-6.

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