

Renin-Angiotensin System in Proliferative Diabetic Retinopathy and Its Gene Expression in Cultured Human Müller Cells

Teruyo Kida*, Tsunehiko Ikeda*,
Masato Nishimura[†], Tetsuya Sugiyama*,
Yutaka Imamura*, Chie Sotozono[‡], Kohji Nishida[‡], Shigeru Kinoshita[‡],
Manabu Yoshimura[†], Kimitoshi Nakamura[§] and Naoki Inokuchi[¶]

**Department of Ophthalmology, Osaka Medical College, Osaka,
Japan; †Departments of †Clinical and Laboratory Medicine, ‡Ophthalmology,
Kyoto Prefectural University of Medicine, Kyoto, Japan; §Nakamura Eye Clinic,
Nagano, Japan; ¶Department of Ophthalmology, Osaka Kaisei Hospital, Osaka, Japan*

Purpose: To determine if the renin-angiotensin system (RAS) is involved in diabetic retinopathy, we measured the levels of angiotensin-converting enzyme (ACE) and angiotensin II in the vitreous of patients with proliferative diabetic retinopathy (PDR). We also investigated whether the genes of the RAS factors were expressed by cultured human Müller cells.

Methods: Vitreous samples were collected during vitreous surgery from patients with PDR, and from patients with idiopathic macular hole, who served as controls. The concentration of ACE was analyzed, and the level of angiotensin II was quantitatively determined by double antibody radioimmunoassay. In addition, the cDNA, prepared from the mRNA extracted from cultured human Müller cells, was used as a template for reverse transcriptase-polymerase chain reaction with primers selected for renin, angiotensinogen, ACE, and angiotensin receptor type I.

Results: The mean concentration of ACE was 0.82 ± 0.73 IU/L at 37°C in the PDR group, which was significantly higher than the 0.05 ± 0.07 IU/L at 37°C in the controls. The mean concentration of angiotensin II was 8.77 ± 4.57 pg/mL in the PDR group, which was significantly higher than the 5.1 ± 1.7 pg/mL in the controls. mRNA for renin, angiotensinogen, ACE, and angiotensin receptor type I was detected in cultured human Müller cells.

Conclusions: The RAS is locally activated in eyes with PDR, and Müller cells may play a role in this local activation. **Jpn J Ophthalmol 2003;47:36–41** © 2003 Japanese Ophthalmological Society

Key Words: Angiotensin II, angiotensin-converting enzyme, Müller cells, proliferative diabetic retinopathy, renin-angiotensin system.

Introduction

The renin-angiotensin system (RAS) plays an important role in the regulation and maintenance of blood pressure, body fluids, and electrolytes. Renin is involved in the conversion of angiotensinogen to angiotensin I, and the angiotensin-converting en-

zyme (ACE) is involved in the conversion of angiotensin I to angiotensin II.¹ Angiotensin II, which is the active substance in RAS, has vasoconstrictive effects.¹ Recent reports have focused on the relationship of angiotensin II to neovascularization.^{2,3}

In addition to its effects on the general circulation, the RAS has local effects in the brain, adrenal glands, gonads, and placenta.^{1,4–6} Moreover, ACE and angiotensin II have been shown to have localized intraocular effects.^{1,2,7} However, much remains unknown regarding the function of the intraocular RAS.

Received: November 19, 2001

Correspondence and reprint requests to: Tsunehiko IKEDA, MD, Department of Ophthalmology, Osaka Medical College, 2-7 Daigaku-cho, Takatsuki, Osaka 569-8686, Japan

Several studies have shown elevated serum ACE levels in patients with diabetes mellitus, and high vitreous concentrations of prorenin and renin in patients with proliferative diabetic retinopathy.⁸⁻¹⁰ These reports suggest a possible association between the RAS and the progression of diabetic retinopathy. In addition, Müller cells, which are the primary glial cells in the neurosensory retina, are reported to produce renin.¹¹ This finding has led to an interest in the relationship between Müller cells and the RAS.

The purpose of this study was to quantify the vitreous concentrations of ACE and angiotensin II in eyes with proliferative diabetic retinopathy, and to investigate the genetic expression of various RAS factors in cultured human Müller cells by reverse transcriptase-polymerase chain reaction (RT-PCR).

Materials and Methods

Collection of Vitreous Humor

Using a vitreous cutter, without intraocular irrigation, 0.6 to 1.0 mL of pure vitreous gel was collected during vitreous surgery. The vitreous samples were frozen and stored at -80°C . Vitreous gel was collected from 29 eyes of 29 patients with proliferative diabetic retinopathy. Of these, 8 eyes had macular tractional retinal detachment (group 1), 15 eyes had extramacular tractional retinal detachment accompanied by vitreous hemorrhage (group 2), and 6 eyes had vitreous hemorrhage only (group 3). Vitreous samples were also collected from a control group of 20 eyes of 20 patients with idiopathic macular holes. Informed consent was obtained from each subject. All experimental procedures conformed to the tenets of the Declaration of Helsinki.

Quantification of ACE and Angiotensin II

Kasahara's method¹² was used to assay for ACE after confirming the linearity of this method. When the thawed sample of vitreous was added to p-hydroxybenzoyl glycine-L-histidyl-L-leucine, the ACE present in the vitreous reacted to produce free p-hydroxybenzoyl glycine. A reagent was then added, and colorimetry was performed (505 nm, 37°C) on the final quinone imine pigment produced to obtain the vitreous concentration of ACE.

Angiotensin II was measured using a double antibody radioimmunoassay. Anti-angiotensin II antibody and a tracer were added to the vitreous samples, and a secondary antibody and polyethylene glycol (PEG) were then used to produce a precipitate. After centrifugation, the radioactivity of the precipitated fraction was measured to obtain a stan-

dard curve from which the concentration of angiotensin II was calculated.

Genetic Expression of Various RAS Factors in Cultured Human Müller Cells

Dr. Donald G. Puro (Kellogg Eye Center, University of Michigan, Ann Arbor, MI, USA) kindly provided the cultured human Müller cells. Cultures were prepared as detailed elsewhere, and were incubated at 36°C in 80% medium A (80% Dulbecco's modified Eagle medium/Ham F12 (Gibco BRL, Gaithersburg, MD, USA) and 20% fetal bovine serum (Hyclone, Logan, UT, USA) containing 200 U/mL penicillin G, 200 $\mu\text{g}/\text{mL}$ streptomycin sulfate, 0.5 g/mL amphotericin B (Gibco) in an atmosphere of 95% humidity and 5% CO_2 . This culture system appeared to be a reasonable one to study the aspects of the pathophysiology of human retinal glial cells because the cells prepared by this method were found to be immunoreactive to an antibody specific for Müller cells and to have morphological features of glial cells in multicellular complexes (ie, the epiretinal and preretinal membranes) located on the retinal surface and extending into the vitreous in eyes with various retinal diseases.

Poly A RNA was extracted from the cultured cells with an mRNA purification kit (Pharmacia-LKB, Uppsala, Sweden). Complementary DNA (cDNA) was generated as described previously. After ethanol precipitation, the mRNA was resuspended in 10 μL of water (2 μL of which had been incubated at 65°C for 5 minutes), and then was chilled on ice, and reverse-transcribed in 10 μL of a solution containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl_2 , 10 mM dithiothreitol, 100 units MoMuLV RNase H-reverse transcriptase, 20 units of RNasin (ribonuclease inhibitor; Toyobo, Osaka), 1 μM of oligo-dT primer, and 200 μM each of dATP, dCTP, and dGTP. This mixture was incubated at 43°C for 1.5 hours, heated to 95°C for 10 minutes, and then stored at -20°C . Next, specific sense and antisense primers were prepared for various RAS factors, including renin, angiotensinogen, ACE, and angiotensin type I receptors (Table 1). The specific genes for each were amplified by RT-PCR. cDNA products (5 μL) were added to a 50- μL reaction mixture containing 5 μL of $10\times$ PCR reaction buffer ([500 mM KCl, 100 mM Tris-HCl [pH 8.8], 15 mM MgCl_2 , 1% Triton X-100, 200 nM of each primer, 1.0 unit of *Taq* DNA polymerase [Takara, Kyoto], and 200 μM each of dATP, dCTP, dGTP, and dTTP).

Reaction mixtures were prepared for multiple samples and divided into aliquots. A negative con-

Table 1. Primers for Polymerase Chain Reaction*

Renin mRNA		
5' primer	AAATGAAGGGGGTGTCTGTGG	(bases 851–872)
3' primer	AAGCCAATGGGGTTGTTACGC	(bases 1206–1227)
Angiotensinogen mRNA		
5' primer	CTGCAAGGATCTTATGACCTGC	(bases 1209–1231)
3' primer	TACACAGCAAACAGGAATGGGC	(bases 1404–1426)
ACE mRNA		
5' primer	GCCTCCCAACAAGACTGCCA	(bases 492–512)
3' primer	CCACATGTCTCCAGCCAGATG	(bases 860–880)
Angiotensinogen type I receptors mRNA		
5' primer	GGCCAGTGTTCCTTTTGAATTTAGCAC	(bases 186–214)
3' primer	TGAACAATAGCCAGGTATCGATCAATGC	(bases 368–395)
GAPDH mRNA		
5' primer	CCCATCACCATCTCCAGGAG	(bases 211–231)
3' primer	GTTGTCATGGATGACCTTGGC	(bases 475–495)

*Specific sense and antisense primers were prepared for various renin-angiotensin system factors, including renin, angiotensinogen, angiotensin-converting enzyme, and angiotensin type I receptors.

trol consisting of a 50- μ L aliquot without cDNA was included in each amplification. After 25 μ L of mineral oil was layered over the aqueous phase to prevent evaporation, amplification was performed for 45 cycles using a DNA thermal cycler. For renin, ACE and angiotensin type I receptors, each cycle consisted of 30 seconds at 95°C for denaturation, 30 seconds at 62°C for annealing, and 1 minute at 72°C for primer extension. For angiotensinogen, each cycle consisted of 30 seconds at 95°C for denaturation, 1 minute at 55°C for annealing, and 1 minute at 72°C for primer extension.

The PCR products were electrophoresed using 1.5% agarose gel, then colored with ethidium bromide. Sequencing of PCR products was performed with an auto DNA sequencer using a PRISM DyeDeoxy Terminator cycle sequencing kit. Negative controls were performed using distilled water instead of RNA. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as a positive control.

Results

The mean level of ACE in the vitreous was 0.82 ± 0.73 U/L at 37°C for the diabetic retinopathy group and 0.05 ± 0.07 U/L at 37°C for the idiopathic macular hole group (Figure 1). The mean level in the diabetic retinopathy group was significantly higher than in the idiopathic macular hole group ($P = .0001$). The mean level of ACE for each proliferative diabetic retinopathy subgroup was as follows: 0.92 ± 0.71 IU/L at 37°C in group 1; 0.71 ± 0.66 IU/L at 37°C in group 2; and 0.85 ± 0.82 IU/L at 37°C in group 3. The differences in the mean levels in the three subgroups were not significant.

For angiotensin II, the mean level was 8.77 ± 4.57 pg/mL in the diabetic retinopathy group and 5.1 ± 1.7 pg/mL in the idiopathic macular hole group (Figure 2). The mean level of angiotensin II was significantly higher in the diabetic retinopathy group ($P = .0121$). The level for each proliferative diabetic retinopathy subgroup was as follows: 9.10 ± 3.99 pg/mL in group 1; 8.70 ± 5.11 pg/mL in group 2; and 8.46 ± 4.69 pg/mL in group 3. The differences between these subgroups were not significant.

The results of the RT-PCR for the various RAS factors in the cultured human Müller cells are shown

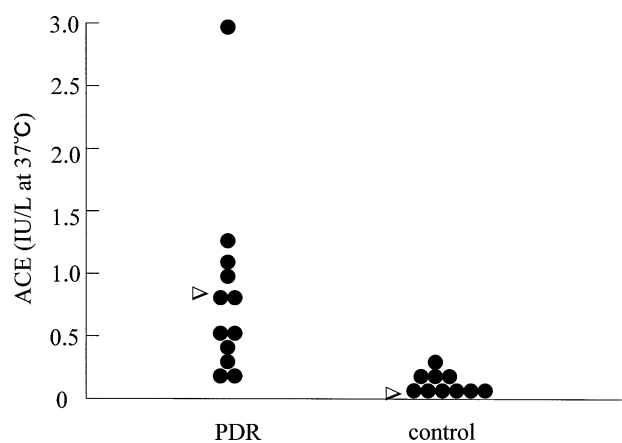


Figure 1. Results of assays for angiotensin-converting enzyme (ACE) in the proliferative diabetic retinopathy (PDR) group of patients ($n = 12$) and in the idiopathic macular hole group of patients ($n = 10$) who served as controls. The mean value for each group is indicated by the arrowhead. The levels are significantly higher in the PDR group ($P = .0001$, Mann-Whitney U -test).

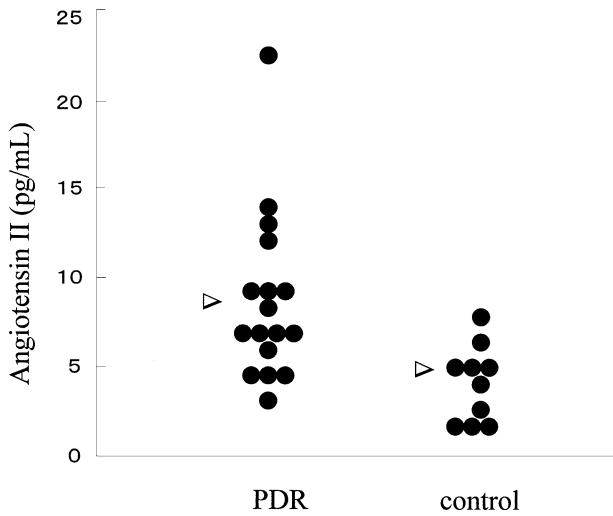


Figure 2. Results of the assay for angiotensin II in the proliferative diabetic retinopathy (PDR) group ($n = 17$) and in the idiopathic macular hole group ($n = 10$). The mean value for each group is indicated by an arrowhead. As with the angiotensin-converting enzyme concentrations, the levels of angiotensin II are significantly higher in the PDR group ($P = .0121$, Mann-Whitney U -test).

in Figure 3. Gene expression was detected in the cultured human Müller cells for renin, angiotensinogen, ACE, and angiotensin type I receptors. In contrast, we confirmed that there was no product in the negative control (data not shown). GAPDH was used as the positive control.

Discussion

Recent advances in molecular biology have enabled researchers to detect and localize RAS gene expression in various tissues.^{1,4-6,13,14} These studies have shown that tissue specificity plays a role in regulating this gene expression. Thus, the functional and pathophysiological aspects of the RAS have been investigated both in the general circulation and in individual tissues.

Several studies have reported the gene expression of various RAS factors in ocular tissues. Danser et al⁷ reported that the concentrations of prorenin and renin were higher in the human vitreous and anterior chamber than in the serum. They concluded that these substances might be produced locally in these ocular tissues. Furthermore, the vitreous concentrations of these substances were even higher in patients with proliferative diabetic retinopathy.^{7,8} Thus, these RAS factors might be associated with the progression of diabetic retinopathy. In a study on enucleated human eyes, Wagner et al¹ detected renin

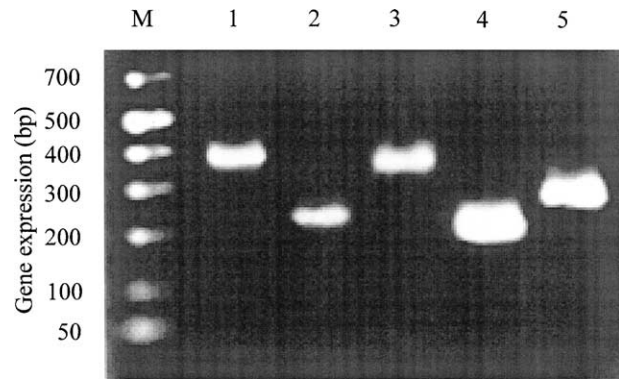


Figure 3. Reverse transcriptase-polymerase chain reaction results for various renin-angiotensin system factors in cultured human Müller cells. Glyceraldehyde 3 phosphate dehydrogenase (5) was used as the positive control. Gene expression (bp) is present in the cultured human Müller cells for renin (1), angiotensinogen (2), angiotensin-converting enzyme (3), and angiotensin type I receptors (4). M: markers.

mRNA in the retinal pigment epithelial cells, choroid, and neurosensory retina. They also identified gene expression for angiotensinogen and ACE in the choroid and neurosensory retina, as well as for angiotensin II, which was found in particularly high concentrations in vascular tissues.

Angiotensin II, a physiologically active substance in the RAS, is known to have vasoconstrictive effects.¹⁵ Recent investigations have also focused on the angiogenic effects of this substance. Fernandez et al³ noted that angiotensin II induced into corneal pockets produced corneal neovascularization. King et al^{16,17} observed that angiotensin II induced the proliferation of cultured aortic endothelial cells. In an in vitro study, Takagi et al¹⁸⁻²⁰ found that angiotensin II caused a time-dependent increase in the expression of KDR mRNA, a vascular endothelial growth factor (VEGF) receptor subtype. The results of this latter study suggest that angiotensin II may act as an exacerbating factor in the development of VEGF-dependent ischemic neovascularization in patients with proliferative diabetic retinopathy. Danser et al⁸ found that the vitreous concentrations of prorenin and renin were higher in patients with proliferative diabetic retinopathy than in controls. Thus, other factors besides angiotensin II may also be associated with the progression of diabetic retinopathy.

These studies identified the gene expression for various RAS factors in ocular tissues, including the retina, which may play important roles in the progression of proliferative diabetic retinopathy, espe-

cially with regard to neovascularization. In the present study, the concentrations of ACE and angiotensin II were found to be significantly higher in the vitreous samples obtained from patients with proliferative diabetic retinopathy than in those from control patients with idiopathic macular holes. To the best of our knowledge, our study is the first to report the detection of the enzymatic activity of ACE in the vitreous of eyes with diabetic retinopathy. This is in agreement with the studies mentioned above.^{7–9} Endo et al²¹ found that total protein concentrations were three times higher in the vitreous of diabetic eyes than those in controls. We calculated the ACE and angiotensin II levels/total protein concentration in vitreous, and then, the corrected level was significantly higher in the vitreous of eyes with proliferative diabetic retinopathy than in controls.

Our study also examined the vitreous concentrations of these substances in three established subgroups, stratified on the basis of disease severity within the proliferative diabetic retinopathy group, but there were no significant differences among these subgroups. However, it should be noted that the patients in these three subgroups all had relatively severe conditions as evidenced by the presence of intraocular neovascularization. Further study is needed to determine how the vitreous concentrations of these RAS factors, ie, ACE and angiotensin II, might differ between patients with proliferative diabetic retinopathy and those with simple diabetic retinopathy.

It has not been determined whether renin is expressed in the retinal vasculature or in the neuronal or glial cells (Müller cells) of retina. However, Kumar et al studied rat brain samples and found elevated angiotensinogen levels in neurons and glial cells and elevated ACE levels in neurons. Berka et al¹¹ reported that renin is specifically localized in the Müller cells from humans, BALB/c mice, and Sprague-Dawley rats. They also stated that their morphologic and biosynthetic findings were consistent with renin synthesis within Müller cells. The Müller cells, which are the predominant glial cells in the neurosensory retina, are a probable source of various cell growth factors induced by retinal ischemia including VEGF. Therefore, it is likely that these cells play an important role in the pathophysiological development of neovascularization in patients with proliferative diabetic retinopathy. Thus, we believe that Müller cells, like brain glial cells, may also be a source for various RAS factors.

The present study utilized RT-PCR to investigate the genetic expression of various RAS factors in cul-

tured human Müller cells. Gene expressions of renin, angiotensinogen, ACE, and angiotensin type I receptors were detected. To the best of our knowledge, this is the first report of the genetic expression of angiotensin type I receptors in cultured human Müller cells. These findings suggest that RAS is locally activated in PDR, and that the Müller cells may play a role in this local activation in the eye.

The origin of high ACE and angiotensin II levels in vitreous will be much clearer if we study the Müller cells of diabetic patients, or study Müller cells exposed to high glucose in the low pathological levels. However, we could not use Müller cells obtained from diabetic patients in this study because using human-donor eyes for research is prohibited in Japan.

Other recent studies have suggested that the administration of ACE inhibitors can be useful for the treatment of diabetic retinopathy.^{22,23} The suppression of angiotensin II production by ACE inhibitors is useful for blood pressure control and improving retinal circulation. In addition, inhibition of angiotensin II production may indirectly inhibit intraocular neovascularization.²⁴ Additional studies are required to confirm the latter effect. Further detailed investigations regarding the relationship of the RAS to the pathophysiology of diabetic retinopathy will likely result in the development of new treatment options for diabetic patients with retinopathy. Further studies will also be needed to investigate RAS expression in Müller cells at the protein level, as well as to examine pathogenic changes in these cells due to hypoxemia.

References

1. Wagner J, Danser AHJ, Derkx FHM, et al. Demonstration of renin mRNA, angiotensinogen mRNA, and angiotensin converting enzyme mRNA expression in the human eye: evidence for an intraocular renin-angiotensin system. *Br J Ophthalmol* 1996;80:159–163.
2. Sramek SJ, Wallow IHL, Tewksbury DA, et al. An ocular renin-angiotensin system. Immunohistochemistry of angiotensinogen. *Invest Ophthalmol Vis Sci* 1992;33:1627–1632.
3. Fernandez LA, Twickler J, Mead A. Neovascularization produced by angiotensin II. *J Lab Clin Med* 1985;105:141–145.
4. Kumar A, Rassoli A, Raizada MK. Angiotensinogen gene expression in neuronal and glial cells in primary cultures of rat brain. *J Neurosci* 1988;19:287–290.
5. Dzau VJ, Ingelfinger JR, Pratt RE. Regulation of tissue renin and angiotensin gene expressions. *J Cardiovasc Pharmacol* 1986;8(Suppl 10):11–16.
6. Paul M, Wagner J, Dzau VJ. Gene expression of the renin-angiotensin systems in human tissues: quantitative analysis by the polymerase chain reaction. *J Clin Invest* 1993;91:2058–2064.
7. Danser AHJ, Derkx FHM, Admiraal PJJ, et al. Angiotensin levels in the eye. *Invest Ophthalmol Vis Sci* 1994;35:1008–1018.

8. Danser AHJ, van den Dorpel MA, Deinum J, et al. Renin, prorenin, and immunoreactive renin in vitreous fluid from eyes with and without diabetic retinopathy. *J Clin Endocrinol Metab* 1989;68:160–167.
9. Ferrari-Dileo G, Ryan JW, Rockwood EJ, et al. Angiotensin-converting enzyme in bovine, feline, and human ocular tissues. *Invest Ophthalmol Vis Sci* 1988;29:876–881.
10. Kohner EM. The renin-angiotensin system and diabetic retinopathy. *Klin Wochenschr* 1992;69(Suppl XXIX):25–27.
11. Berka JL, Stubbs AJ, Wang DZM, et al. Renin-containing Müller cells of the retina display endocrine features. *Invest Ophthalmol Vis Sci* 1995;36:1450–1458.
12. Kasahara Y, Ashihara Y. Colorimetry of angiotensin I converting enzyme activity in serum. *Clin Chem* 1981;27:1922–1925.
13. Nagi DK, Foy CA, Mohamed Ali V, et al. Angiotensin I converting enzyme (ACE) gene polymorphism, plasma ACE levels and their association with the metabolic syndrome and electrocardiographic coronary artery disease in Pima Indians. *Metabolism* 1998;47:622–626.
14. Nagi DK, Mansfield MW, Stickland MH, Grant PJ. Angiotensin converting enzyme (ACE) insertion/deletion (I/D) polymorphism, and diabetic retinopathy in subjects with IDDM and NIDDM. *Diabet Med* 1995;12:997–1001.
15. Ferrari-Dileo G, Davis EB, Anderson DR. Angiotensin II binding receptors in retinal and optic nerve head blood vessels. An autoradiographic approach. *Invest Ophthalmol Vis Sci* 1991;32:21–26.
16. King SJ, Bech JC, Harding JW, Hosick HC. Growth promoting effects of angiotensins on cultured bovine aortic endothelial cells. *J Cell Biol* 1986;103:299.
17. Feener EP, Northrop JM, Aiello LP, King GL. Angiotensin II induces plasminogen activator inhibitor-1 and -2 expression in vascular endothelial and smooth muscle cells. *J Clin Invest* 1995;95:1353–1362.
18. Takagi H, King GL, Aiello LP. Identification and characterization of vascular endothelial growth factor receptor (Flt) in bovine retinal pericytes. *Diabetes* 1996;45:1016–1023.
19. Otani A, Takagi H, Suzuma K, Honda Y. Angiotensin II potentiates vascular endothelial growth factor-induced angiogenic activity in retinal microcapillary endothelial cells. *Circ Res* 1998;82:619–628.
20. Otani A, Takagi H, Oh H, et al. Angiotensin II-stimulated vascular endothelial growth factor expression in bovine retinal pericytes. *Invest Ophthalmol Vis Sci* 2000;41:1192–1199.
21. Endo H, Naito T, Asahara T, et al. Cytokines in the vitreous fluid of patients with proliferative diabetic retinopathy: vascular endothelial growth factor and platelet-derived growth factor are elevated in proliferative diabetic retinopathy. *Nippon Ganka Gakkai Zasshi (J Jpn Ophthalmol Soc)* 2000;104:711–716.
22. Jackson WE, Holmes DL, Garg SK, et al. Angiotensin-converting enzyme inhibitor therapy and diabetic retinopathy. *Ann Ophthalmol* 1992;24:99–103.
23. Chaturvedi N, Sjolie AK, Stephenson JM, et al. Effect of lisinopril on progression of retinopathy in normotensive people with type 1 diabetes. *Lancet* 1998;351:28–31.
24. Paolisso G, Tagliamonte MR, Gambardella A, et al. Losartan mediated improvement in insulin action is mainly due to an increase in nonoxidative glucose metabolism and blood flow in insulin-resistant hypertensive subjects. *J Hum Hypertens* 1997;11:307–312.