

Increased Expression of Angiotensin-converting Enzyme in Retinas of Diabetic Rats

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Purpose: The aim of this study was to examine the localization and the changes in the amount of angiotensin-converting enzyme (ACE) and the relationship between the renin-angiotensin (RA) system and vascular endothelial growth factor (VEGF)/VEGF-receptor system in the retinas of diabetic rats.

Methods: Immunohistochemical localization of ACE, VEGF, and VEGF-receptor fetal liver kinase-1 (Flk-1) was examined in cryosections of the retinas of streptozotocin-injected diabetic rats. A semi-quantitative comparison of diabetic rats with age-matched controls was also performed by counting the ACE- or Flk-1-positive vessels per microscopic field.

Results: ACE immunoreactivity was localized in the retinal vessel walls, and the percentages of ACE-positive vessels were significantly increased in the retinas of diabetic rats maintained 3 to 5 months. Both VEGF and Flk-1 signals increased simultaneously with the increment of ACE immunoreactivity.

Conclusions: ACE, expressed in the retinal vessel walls, increases simultaneously with the increment of both VEGF and Flk-1 in the retinas of diabetic rats, suggesting that upregulation of ACE might play some role in the progression of diabetic retinopathy through the VEGF/VEGF receptor system. **Jpn J Ophthalmol 2001;45:585-591** © 2001 Japanese Ophthalmological Society

Key Words: Angiotensin-converting enzyme, diabetic retinopathy, renin-angiotensin system, retinal vessels, streptozotocin.

Introduction

The renin-angiotensin (RA) system plays an important role in the control of systemic blood pressure and electrolyte homeostasis. In the RA system, angiotensin-converting enzyme (ACE), abundantly expressed in the pulmonary vascular endothelium, is a crucial enzyme that converts an inactive peptide,

angiotensin I, into a potent vasopressor octapeptide, angiotensin II.¹ The RA system exists both in the circulating blood and in several types of tissue, including the eyes,¹⁻³ thus suggesting that the local RA system plays an important role in the homeostasis of ocular tissue.

Recently, the RA system has been shown to play an important role in the progression of diabetic complications; for example, the progression of diabetic retinopathy has been shown to be associated with elevated serum ACE.⁴ Accordingly, the RA system is assumed to be one of the key systems in the development of diabetic retinopathy. In 1979, Ward et al⁵ re-

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ported for the first time that ACE has a strong biological activity in isolated pig retinal vessels. Subsequently, many researchers reported the presence of ACE in the retina of many species while also describing that the biological activity of ACE and the mRNA of ACE were found in the whole retina.^{6,7} However, the localization of ACE in the retina has not yet been reported. In this connection, the expression of ACE in the ocular RA system of individuals with diabetes mellitus (DM) or non-DM seems to be of great significance in order to clarify the role of the RA system in diabetic retinopathy.

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific angiogenic and permeability-inducing factor^{8,9} that has been implicated in several retinal disorders, including diabetic retinopathy, and clinical studies have shown a relationship between proliferative retinopathy and VEGF levels in ocular fluid.¹⁰ Furthermore, VEGF and fetal liver kinase-1 (Flk-1), one of the VEGF receptors, have increased in retinas of streptozotocin (STZ)-induced diabetic rats and expressed as nonproliferative retinopathy.^{11,12}

In this study, we have attempted to clarify the localization and quantitative alteration of ACE in the retinas of STZ-induced diabetic rats, and to elucidate the correlation between the RA system and the VEGF/VEGF receptor system under these conditions by the use of immunohistochemical techniques.

Materials and Methods

Animal Treatment

All animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and were also reviewed and accepted by the Ethics Committee on Animal Experiments, Faculty of Medicine, Kyushu University. Diabetic rats were obtained as previously described.¹³ Briefly, 52 Wistar rats each weighing approximately 200 g (Kyudo, Kumamoto) were intravenously injected with either STZ (65 mg/kg body weight dissolved in 0.1 M citrate buffer, pH 4.5) (diabetic group, $n = 26$) or with 0.1 M citrate buffer (nondiabetic group, $n = 26$). The development of diabetes was confirmed by persistent hyperglycemia (>400 mg/dL), polyuria, and impaired growth. After the injection of STZ, the diabetic rats were sacrificed with an overdose of sodium pentobarbital (200 mg/kg) at 1 month (7 rats), 3 months (10 rats), 4 months (1 rat), and 5 months (8 rats). The same numbers of nondiabetic rats were sacrificed at the end of each period.

Immunohistochemical Studies

Fresh eyes were cut in half along the equator, and their posterior sides were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) on ice for 2 hours. After incubation with 30% sucrose in PBS at 4°C overnight, the eyes were rapidly frozen in liquid nitrogen-cooled OCT compound (Miles, Elkhart, IN, USA). Frozen sections (8 μ m thick) were incubated in absolute acetone at room temperature for 10 minutes and then were reacted with monoclonal mouse anti-ACE antibody^{14,15} (9B9; Chemicon International, CA, USA) diluted 1:1000, polyclonal rabbit anti-von Willebrand factor (vWF) antibody (DAKO A/S, Glostrup, Denmark) diluted 1:800, polyclonal rabbit anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) concentrated at 1 μ g/mL, or polyclonal rabbit anti-Flk-1 antibody (Santa Cruz Biotechnology) concentrated at 1 μ g/mL. Immunoreactivity was detected by the avidin-biotin complex immunoperoxidase method¹⁶ using 3-amino-9-ethyl carbazole (AEC; DAKO, Carpinteria, CA, USA) as the chromogen. All sections were counterstained with hematoxylin.

Statistical Analysis of

Percentage of ACE-positive Vessels

Each pair of serial sections obtained from diabetic rats at 1 month (14 eyes from 7 rats), 3 months (15 eyes from 8 rats), and 5 months (6 eyes from 6 rats), or from the same numbers of age-matched controls was immunohistochemically stained for ACE or vWF. Subsequently, three corresponding areas of the retinas (posterior, mid-peripheral, and peripheral areas) were examined under a microscope at high-power fields. Masked observers (IY and TS) counted the number of ACE- and vWF-positive vessels per $\times 200$ microscopic field. The data from each eye were expressed as the mean of the nine areas from the three pairs of sections. The percentage of ACE-positive vessels was determined by the following expression: The percentage of ACE-positive vessels = (ACE-positive vessels)/(vWF-positive vessels) $\times 100$.

The data obtained from the eyes in each group were then analyzed using the unpaired *t*-test. $P < .05$ was considered to be significant.

Statistical Analysis of Number

of Flk-1-positive Vessels in Rat Retinas

The sections obtained from 20 eyes of 20 rats (diabetes for 3 months: 5 eyes from 5 rats, 5 months: 5 eyes from 5 rats, and the same numbers of age-

matched controls) were immunostained for Flk-1. Three areas of the retinas (posterior, mid-peripheral, and peripheral areas) were chosen and the number of Flk-1-positive vessels per $\times 400$ microscopic field was counted. The data from each rat was expressed as the mean of the nine areas from the three sections. The data obtained from the rats in each group were then analyzed using the unpaired *t*-test. $P < .05$ was considered to be significant.

Statistical Analysis of Correlation of Quantitative Alteration Between ACE and Flk-1 in Diabetic Rats

Fifteen pairs of serial sections obtained from 5 eyes of 5 rats in each group were immunohistochemically stained for ACE and Flk-1. Three corresponding areas of the retinas (posterior, mid-peripheral, and peripheral areas) were chosen and the number of ACE- and Flk-1-positive vessels per $\times 400$ microscopic field was counted. All data were presented as the number of ACE- or Flk-1-positive vessels in a microscopic field, and the correlation of quantitative alteration between ACE and Flk-1 was then analyzed using the Pearson correlation analysis.

Results

Localization of ACE in Rat Retina

In the normal rat retinas, faint immunoreactivity to ACE was mainly localized in the nerve fiber, inner nuclear and outer plexiform layers, and immunoreactivity seemed to be present in the retinal vessels (Figure 1A). The specificity of this stain was confirmed by the absence of staining in control mouse IgG (data not shown). However, in the STZ-induced diabetic rats maintained for 5 months, the immunoreactivity to ACE significantly increased in both intensity and number of stained vessels (Figure 1B). To identify the localization of ACE in the retinas collected from the diabetic group, in serial sections we performed an immunohistochemical analysis for ACE and vWF, which is a specific marker for vascular endothelial cells. The ACE immunoreactivity seemed to correspond to that of the vascular wall (Figures 1C, D).

Quantitative Alteration of ACE in Retinas of Diabetic Rats

We determined the percentage of ACE-positive vessels in the diabetic group and nondiabetic group, using serial sections immunostained for vWF or ACE (as shown in Figures 1C, D). The ACE-posi-

tive vessels in the nondiabetic group were constantly around 20% regardless of age, while the percentage in the diabetic group significantly increased in direct correlation to the increased duration of DM (Figure 2). No significant difference was observed for the percentage of ACE-positive vessels between the diabetic rats maintained for 1 month and the age-matched rats ($21.3 \pm 4.3\%$ vs $16.1 \pm 2.2\%$, $P = .295$). However, in the diabetic rats maintained for 3 months and 5 months, the percentage of ACE-positive vessels was approximately twice ($56.5 \pm 2.7\%$ vs $26.5 \pm 2.6\%$, $P < .001$) and four times ($83.1 \pm 5.1\%$ vs $18.3 \pm 3.0\%$, $P < .001$) as much as that of the age-matched control rats, respectively. In both diabetic and nondiabetic groups, the percentage of ACE-positive vessels in the peripheral area was obviously less than in the posterior area. In each section, there was no difference in the number of vWF-positive vessels between the diabetic and nondiabetic rats.

VEGF and Flk-1 Immunoreactivities

In the retinas of nondiabetic rats, a very faint immunoreactivity to VEGF was observed only in very few capillaries in the inner nuclear layer (Figure 3A), while Flk-1 immunoreactivity was never seen in the vessels and the other tissues of the retina (Figure 3B). The specificity of this stain was confirmed by the absence of staining with control rabbit serum (data not shown). However, in the diabetic rats maintained for 4 months, intense immunoreactivity to both VEGF (Figure 3D) and Flk-1 (Figure 3E) was seen in the retina. Immunoreactivity to VEGF was localized in the ganglion cell layer and inner nuclear layer and, particularly, some of the blood vessels in the ganglion cell layer were intensively immunostained for VEGF (Figure 3D). Immunoreactivity to Flk-1 was localized in the vWF-positive capillaries of the inner nuclear layer and in the outer plexiform layer (Figures 3E, F).

Correlation Between Flk-1 and ACE in Diabetic Rats

To clarify the relationship between the increase in immunoreactivity of both ACE and Flk-1 in the retinas of diabetic rats, serial sections were immunostained for those antigens. ACE- and Flk-1 immunoreactivity simultaneously increased in the diabetic rat retinas (Figures 4A, B). In the retinas of diabetic rats maintained for 3 months and 5 months, the number of Flk-1-positive vessels was approximately 3 times (2.69 ± 0.93 vs 0.22 ± 0.10 , $P = .056$) and 10 times (8.27 ± 1.57 vs 0.82 ± 0.39 , $P = .0075$) that of age-matched control rats, respectively (Figure 5).

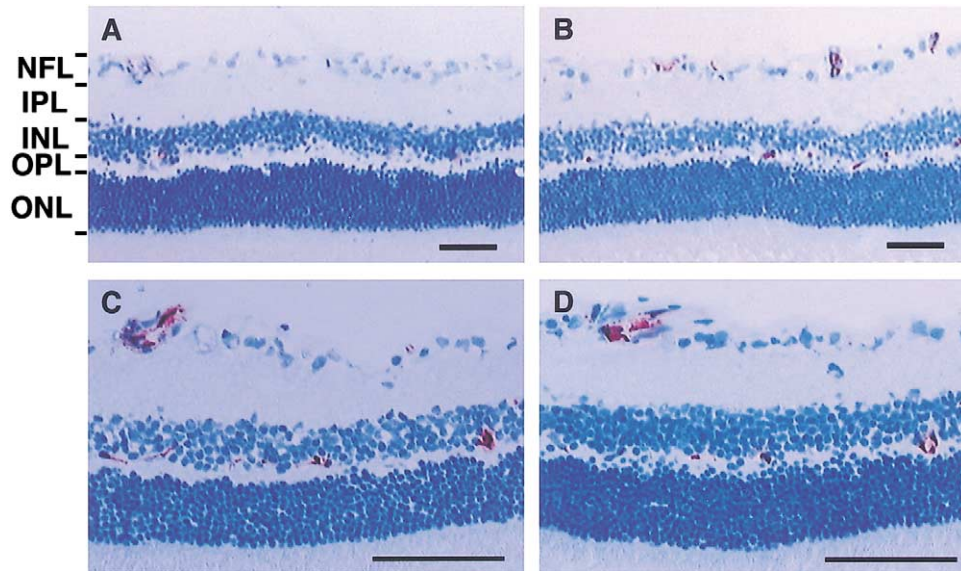


Figure 1. Immunohistochemical micrographs of rat retina. Immunoreactivity to angiotensin-converting enzyme (ACE) (red color) was detected in retinas of nondiabetic (A) and diabetic rats (B). Intense immunoreactivity to ACE was observed in diabetic rat retinas. Serial sections of diabetic rat retina were immunostained for von Willebrand Factor (C) or ACE (D). (A) Nondiabetic rat; (B, C, and D) Diabetic rat maintained for 5 months. NFL: nerve fiber layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer. Bars = 30 μ m.

To correlate the increment of ACE immunoreactivity with that of Flk-1, we counted the numbers of ACE- and Flk-1-positive vessels in the retinas of diabetic rats maintained for 3 and 5 months (Figure 6).

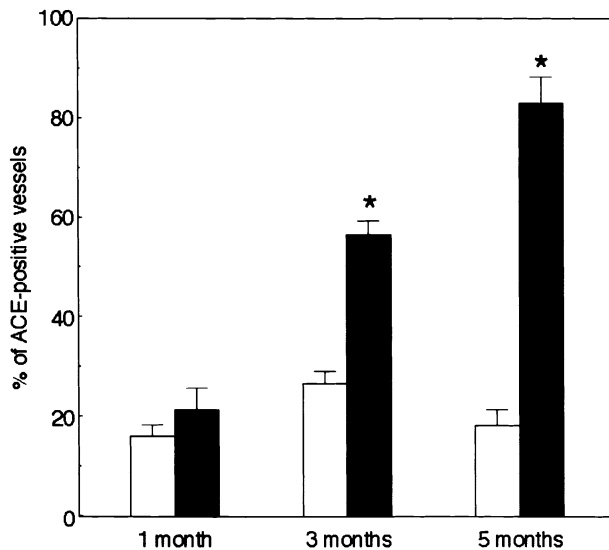


Figure 2. Percentage of angiotensin-converting enzyme (ACE)-positive vessels in rat retinas. Open column: nondiabetes, filled column: diabetes. Columns represent mean \pm SEM. * $P < .001$ difference versus control (unpaired t -test). 1 month, $n = 14$; 3 months, $n = 15$; 5 months, $n = 6$.

There was a strong positive correlation between ACE- and Flk-1-positive vessels in the retinas of diabetic rats maintained for 3 months (Pearson $r = 0.839$, $P < .05$), and a positive correlation in the retinas of diabetic rats maintained for 5 months (Pearson $r = 0.548$, $P < .05$).

Discussion

In this study, we showed the localization of ACE in the retinal vessel walls in normal rats and the increased expression of ACE simultaneously with up-regulation of the VEGF/VEGF receptor system in the retinas of diabetic rats. We showed the first clear evidence that ACE immunoreactivity is present in the normal retinal vessel walls, in contrast to the findings of a previous report.¹⁷ We cannot understand the exact reason why the previous study showed that ACE was undetectable in normal retina. However, it is likely that some difference in the primary antibodies might have affected the results. The monoclonal antibody (9B9) used in this study has been proven to be highly specific to ACE and it has also been used widely by many researchers.^{14,15}

The extent of ACE immunoreactivity in retinal vessel walls significantly increased in STZ-rats. Although immunohistochemical techniques are not always ideal to evaluate the exact amount of immunoreactivity in each tissue, the amount of ACE

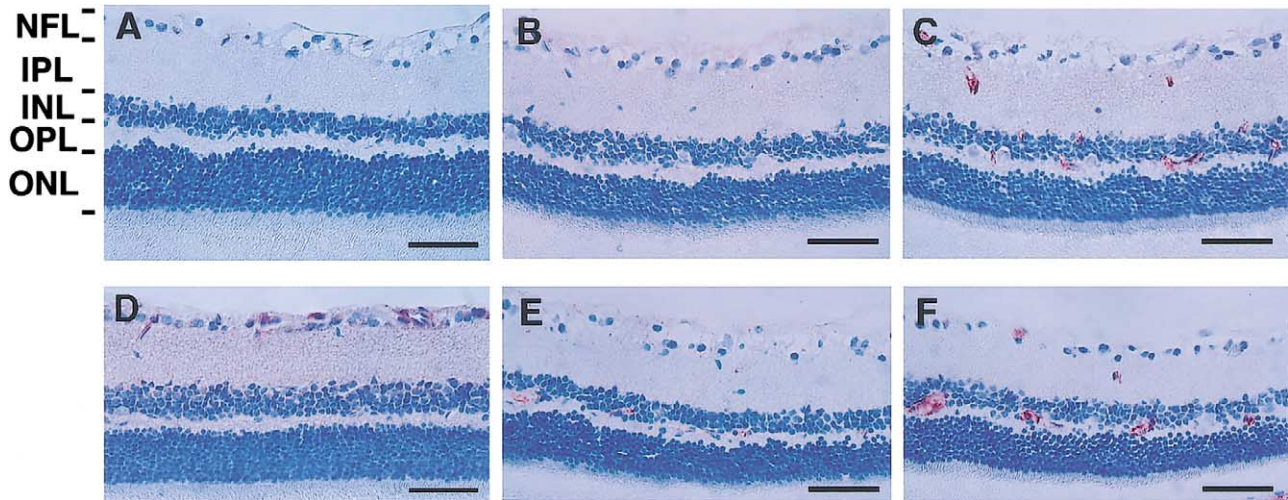


Figure 3. Immunohistochemical micrographs of rat retina stained for and vascular endothelial growth factor (VEGF), fetal liver kinase-1 (Flk-1), and anti-von Willebrand factor (vWF). Immunohistochemical staining for VEGF (A, D), Flk-1 (B, E), and vWF (C, F) was performed in sections of retinas of age-matched nondiabetic control (A, B, C) and diabetic rats maintained for 4 months (D, E, F). Frozen sections (A, D) and two pairs of serial frozen sections (B–C and E–F) were immunostained. NFL: nerve fiber layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer. Bars = 30 μ m.

tended to be higher in the diabetic rat retina than in normal rats. The present experimental procedures were performed carefully and the semi-quantification was done by masked observers. Inter-observer variability was low and the trend of results was reproducible.

In previous reports, the biological activity of ACE in the whole retina of STZ-rats and Zucker fatty diabetic rats decreased significantly in accordance with the progression of diabetic retinopathy, and thus their observations were different from our results.⁶ In order to explain this discrepancy, we considered the following possibility. We found an increase of

ACE immunoreactivity in the vessel walls but no ACE was found in the neural retina of both diabetic and nondiabetic animals. Jacobi et al¹⁸ reported that angiotensin II acted as a neurotransmitter or a neuromodulator in the neural system including the retina, while, in addition, the light signaling system in the retina was also greatly influenced by ACE inhibitor. As a result, they concluded that ACE must exist in the neural retina. Indeed, there might be many factors that masked the immunohistochemical localization of ACE in the neural retina in our study; eg, the amount of ACE in the neural retina might be too small to be detected by the present immunohis-

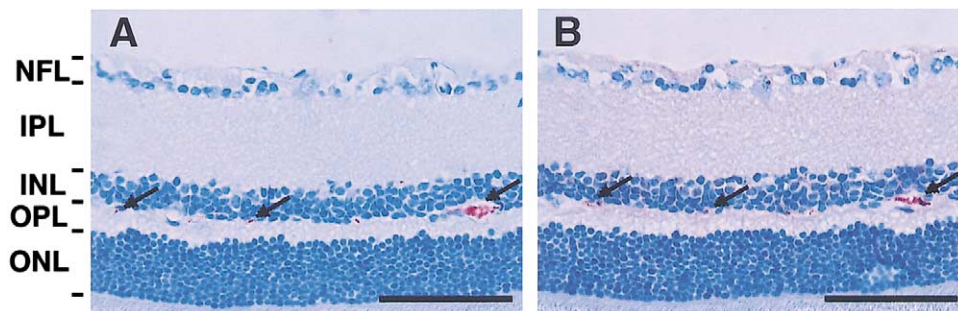


Figure 4. Immunohistochemical micrographs of diabetic rat retina stained for angiotensin-converting enzyme (ACE) and fetal liver kinase-1 (Flk-1). Immunohistochemical staining for ACE (A) and Flk-1 (B) was performed in serial sections of retinas of diabetic rats maintained for 3 months. In high proportion of Flk-1-positive retinal vessels, strong ACE immunoreactivity was observed (arrows). NFL: nerve fiber layer, IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer. Bars = 30 μ m.

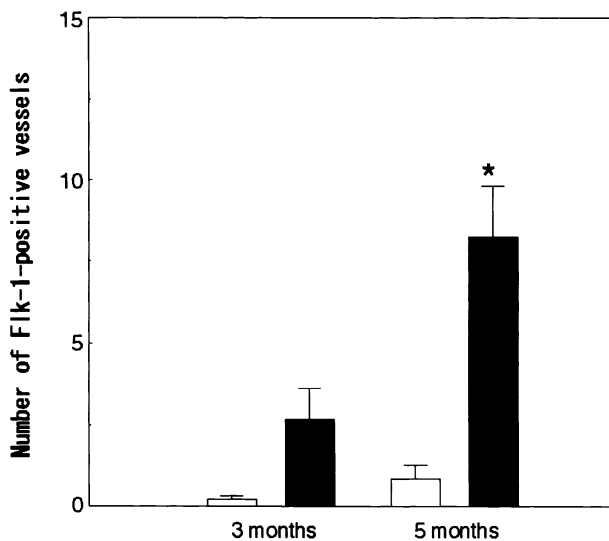


Figure 5. Number of and fetal liver kinase-1 (Flk-1)-positive vessels in diabetic rat retinas. Number of Flk-1-positive vessels increased as diabetes mellitus duration progressed. Open column: nondiabetes, filled column: diabetes. Columns represent mean \pm SEM. * $P < .01$ difference vs age-matched control (unpaired *t*-test). $n = 5$ in each group.

tochemical methods. Furthermore, biological activity in the previous study was measured in the whole retina, including the vessels and neural retina. Therefore, it is not appropriate to compare directly the present data with the previous data. A detailed analysis of the biological activity of ACE in the retina is presently under investigation in our laboratory.

Recently, it has been reported that VEGF and its receptors are increased in STZ-induced diabetic rat

retinas.¹¹⁻¹³ Furthermore, it has been shown that the blood-retinal barrier is disrupted in STZ-induced diabetic rats maintained for more than 3 months¹⁹ and that the blood-retinal barrier breakdown in STZ-induced diabetic rats may be promoted by VEGF.¹³ We have demonstrated the correlation between the increment of Flk-1 and the elevation of ACE, which was accompanied by an increment of VEGF. The correlation coefficient between immunoreactivity for ACE and that of Flk-1 was $r = 0.839$ in 3 months and $r = 0.548$ in 5 months. Flk-1 expression appears to be regulated by various stimuli, including hypoxia^{20,21} and growth factors²²; the increment of Flk-1 may be influenced not only by the RA system but also by many other factors.

Hypertension is one of the major factors involved in the deterioration of diabetic retinopathy.²³ ACE inhibitors, which are most frequently used in current hypertension therapy, have been known to slow the progression of diabetic retinopathy in insulin-dependent DM patients with hypertension.²⁴ These effects are assumed to be due to a lowering of the blood pressure.²⁴ However, important evidence has shown a direct effect of the RA system on diabetic retinopathy.²⁵⁻²⁷ In a clinical study, ACE inhibitor inhibited the breakdown of the blood-retinal barrier and the deterioration of diabetic retinopathy in normotensive insulin-dependent DM patients.²⁵ In this study, we have shown that the increment of ACE in the diabetic rat retina has a relationship to the VEGF/VEGF receptor system. Furthermore, angiotensin II has been reported to stimulate the expression of VEGF receptor and enhance VEGF-induced cell growth and tube formation in cultured bovine

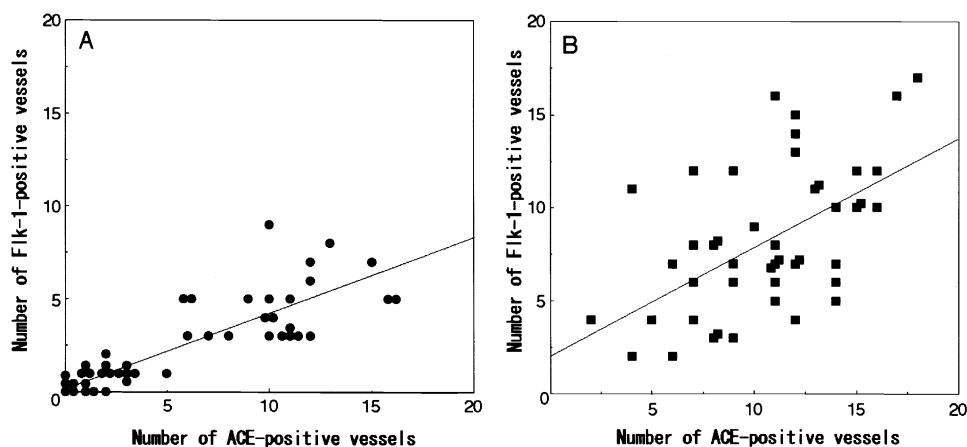


Figure 6. Correlation between angiotensin-converting enzyme (ACE) and fetal liver kinase-1 (Flk-1) with quantitative alteration in retinas of diabetic rats. There was a strong correlation between ACE- and Flk-1-positive vessels in retina of diabetic rats maintained for 3 months (A). Positive correlation was also found in retina of diabetic rat maintained for 5 months (B). (A) Pearson $r = 0.839$ ($P < .05$), (B) $r = 0.548$ ($P < .05$).

retinal vascular endothelial cells,²⁶ to stimulate VEGF expression in bovine retinal pericytes,²⁷ and to induce corneal angiogenesis in vivo.²⁸ If the ACE decreases in the diabetic rat retina, as previous studies described, then ACE inhibitors might not have any protective effect on diabetic retinopathy. ACE inhibitors might prevent the progression of diabetic retinopathy by decreasing the ACE activity in retinal vascular endothelial cells. The local RA system in the retinal vessels thus appears to play a more important role in the progression of diabetic retinopathy.

There are still many unknown factors regarding the local role of the RA system in retinal vasculature, however, the increasing ACE in diabetic rat retinal vessels appears to possibly play a pivotal role in the progression of diabetic retinopathy. The present findings are considered to lead to a better understanding of this disease process as well as to help in the search for new therapeutic modalities.

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