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Alterations in expression of angiopoietins and the Tie-2 receptor in the retina of streptozotocin induced diabetic rats

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Purpose: The angiopoietin (Ang)/Tie-2 system may play a role in vascular integrity and angiogenesis. In this study, we investigated alterations of the gene expression of Ang-1 and Ang-2 in the retinas of streptozotocin (STZ) induced diabetic rats.
Methods: In situ hybridization, reverse transcriptase polymerase chain reaction (RT-PCR) and western blot analyses were performed to determine the mRNA and protein content for Ang-1 and Ang-2 and the Tie2 receptor in the retinas of STZ diabetic and age matched control rats.
Results: Using in situ hybridization analysis, Ang-1, Ang-2, and Tie2 mRNA expression was observed in the ganglion cell layer (GCL) and the inner nuclear layer (INL). While Ang-2 mRNA expression did not changed after 2 weeks, 1 month, or 3 months of STZ induced diabetes, it was increased in the GCL and slightly elevated in the INL after 6 months of diabetes. In contrast, Ang-1 and Tie2 mRNA expression was stable at every timepoint during 6 months of STZ induced diabetes. RT-PCR and western blot analyses confirmed the increase of Ang-2 expression after 6 months of diabetes. Furthermore, double staining of alpha-smooth muscle actin (αSMA) and Ang-2 mRNA demonstrated that the SMA positive cells surrounding Ang-2-expressing cells were decreased in the GCL.
Conclusions: Diabetes increases Ang-2 expression in the GCL accompanied by a reduction of αSMA positive perivascular cells. These changes may suggest a role for Ang-2 in the mechanism of pericyte loss in diabetic retinopathy.

In the retinas of both diabetic humans and diabetic animals, the degeneration and loss of pericytes are important features of morphological abnormality in the microvasculature of diabetic retinopathy [1-3]. Insufficient interaction between pericyte and vascular endothelial cells has recently been reported to cause a retinopathy that mimics diabetic retinopathy, including retinal edema and angiogenesis, hemorrhage, and retinal detachment [4]. This evidence suggests that either degeneration or direct loss of pericytes could contribute to most of the pathological changes observed in the later stages of diabetic retinopathy and is therefore an important phenomenon in understanding diabetic retinopathy. The mechanism of pericyte degeneration, however, is not well understood. High glucose has been reported to induce pericyte loss in vitro through several mechanisms, such as activation of sorbitol pathways [5], protein kinase C [6,7], and glycation end products [8,9]. In in vivo animal models of diabetes, inhibitors of these pathways have been shown to suppress pericyte loss in the retinal vasculature. More direct mechanisms such as activation of oxidative stress [10,11], nuclear factor-kappaB [12], and the proapoptotic effects of the Fas/Fas ligand system have also been suggested to play a role in pericyte loss [13].

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Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) are ligands for the Tie-2 receptor and bind with similar affinity [14,15]. Tie-2 is a member of the endothelium specific receptor tyrosine kinase families [16]. Ang-1 induces the auto-phosphorylation of Tie-2 in cultures of endothelial cells [15], whereas Ang-2 acts as an antagonist and inhibits Ang-1 induced phosphorylation of Tie-2 receptor in vascular endothelial cells [14]. The presence of Ang-2 destabilizes the vessels and it has been proposed that this is a necessary step for angiogenesis, whereas the presence of Ang-1 and the activation of Tie-2 stabilizes vessels. Tie-2-knockout mice die by day 9.5 to 10.5, due to immature vessels and lack of microvessel formation [17,18], although endothelial cell numbers are normal and tubular formation can be detected. A Tie-2 mutation in humans has been reported to cause venous malformations, which are typically an imbalance of endothelial cells and smooth muscle cells [19]. These findings suggest that the Ang and Tie-2 system are the key systems for the endothelial-stromal cell interaction during vascular development. Their role in diabetic retinopathy, however, remains unknown.

In the present study, the effect of diabetes on the retinal Ang/Tie-2 receptor system was investigated. Diabetes increased the gene expression of Ang-2 consistent with a role for this ligand in disruption of the vascular endothelium. However, Ang-1 and Tie-2 gene expression levels remained unchanged. The change in Ang-2 coincided with pericyte loss as determined by immunostaining for smooth muscle actin

(SMA). These data suggest that expression of the Tie-2 antagonist Ang-2 may induce pericyte loss in diabetic retinopathy.

METHODS

Animals: A rat model was used in which diabetes was induced by streptozotocin (STZ; Sigma Chemical, St. Louis, MO). Diabetes was induced in eight Sprague Dawly rats, each eight weeks old, by intravenous injection of 65 mg/kg of STZ in physiologic saline. We confirmed that the plasma glucose level in each rat was greater than 200 mg/dl 48 h later. Eight additional Sprague Dawly rats that were injected with an equal volume of saline alone served as nondiabetic control subjects. All rats were allowed free access to water and food before sacrifice. After injection of STZ, both non-diabetic control rats and diabetic rats were sacrificed and the eyes were enucleated at 2 weeks and at 1, 3, and 6 months. All procedures involving animal experimentation were conducted in accordance with both the guidelines for animal experiments of Kyoto University and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Histochemical samples: Four eye samples were obtained from each group. Briefly, after sacrifice of the rats using an anesthetic overdose, the eyes were obtained and enucleated. The eye samples were then fixed in 4% paraformaldehyde with phosphate buffered saline (PBS) at pH 7.4 for 2 h at 4 °C, dehydrated with a graded alcohol series, and then embedded in paraffin. For paraffin sections, the eyes were serially sectioned at a 5-μm thickness and placed on aminopropyltriethoxysilane coated glass slides (DAKO, Glostrup, Denmark) for in situ hybridization and immunohistochemical staining.

In situ hybridization: cDNA probes for human Ang-1 and Ang-2 were synthesized by reverse transcriptase-polymerase chain reaction (RT-PCR). For Ang-1 and Ang-2 cDNAs, a standard PCR was performed (PCR optimizer kit; Invitrogen, Vienna, Austria) using 5'-AGA ACC ACA CGG CTA CCA TGC T-3' (Ang-1 sense primer corresponding to nucleotides +671 to +692), 5'-TGT GTC CAT CAG CTC CAG TTG C-3' (Ang-1 antisense primer), 5'-AGC TGT GAT CTT GTC TTG GC-3' (Ang-2 sense primer corresponding to nucleotides +377 to +396), 5'-GTT CAA GTC TCG TGG TCT GA-3' (Ang-2 antisense primer corresponding to nucleotides +802 to +821), 5'-GCC TTA ATG AAC CAG CAC CAG G-3' (Tie-2 sense primer corresponding to nucleotides +335 to +356), and 5'-ACT TCT GGG CTT CAC ATC TCC G-3' (Tie-2 antisense primer corresponding to nucleotides +773 to +794) [20]. Sense oligonucleotide probes were used as negative controls. The probes were labeled using a DIG RNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN). Tissue sections (5 μm) were rapidly dewaxed, cleared with alcohol, rehydrated with PBS, pH 7.4, and then digested with Proteinase K (10 mg/mL; Sigma Chemical) for 7 min at 37 °C. The probes were applied in a formamide free diluent, and the slides were heated to 70 °C for 5 min and allowed to hybridize at 45 °C for 16 h. The sections were then washed twice with 2X SSC/50% formaldehyde buffer (1X SSC contains 150 mmol/L NaCl and 15 mmol/L trisodium citrate, pH 7.0) at 45 °C for 1 h and detected with alkaline phosphatase (AP) conjugated anti-digoxigenin antibody. After the hybridization products were washed once in AP chromogen buffer at room temperature, they were visualized with NBT/BCIP (Boehringer Mannheim). The slides were air dried and a coverslip was applied for microscopic examination.

Immunohistochemistry: To observe relationships between pericyte loss and Ang-2, immunohistochemical staining was performed with samples expressing Ang-2 mRNA via in situ hybridization. Sections used for in situ hybridization, were rinsed with PBS. A 0.3% hydrogen peroxide-methanol solution was applied to each specimen for 10 min to block endogenous peroxide activity. After incubating with blocking serum for 20 min, the specimens were incubated overnight at 4 °C with one of the primary antibodies: mouse monoclonal anti-α SMA, 1: 50 dilution (DAKO). Specimens were then washed for 10 min with PBS. A standard indirect immunoperoxidase protocol using the VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA) was performed with 3-amino-9-ethylcarbazole (AEC; DAKO) as the substrate and all incubation steps were performed in a moist chamber. Finally, the slides were washed for 30 min with PBS, and a coverslip was applied with VECTASHIELD (Vector Laboratories) for viewing. As a negative control, normal mouse IgG (DAKO) was used as the primary antibody. Other staining procedures were the same as described earlier.

Semiquantitative reverse transcriptase polymerase chain reaction: Both non-diabetic control rats and diabetic rats were sacrificed and the eyes were enucleated at 2 weeks and at 1, 3, and 6 months. Retinal total RNA was collected by the acid guanidium thiocyanate-phenol chloroform extraction method, as described previously [21]. The primer sequences used were as follows: Ang-1, forward, 5'-CAG CAT CTG GA(A/G) CA(T/C) GT(A/G/T/C) ATG-3'; reverse, 5'-TTC (T/C)TT GTG TTT (A/G/T/C)CC (T/C)TC CAT-3'; Ang-2, forward, 5'-GT(T/G) GA(T/C) TT(T/C) CAG AG(A/G/T/C) AC(A/G/T/C) TGG-3'; reverse, 5'-CGA (A/G)TA GCC (T/G)GA (A/G/T/C)CC (T/C)TT CCA-3' [22]. Normalization of each cDNA concentration was performed using primers for β-actin, 5'-AGC TGA GAG GGA AAT CGT GC-3' (forward) and 5'-ACC AGA CAG CAC TGT GTT GG-3' (reverse) [23]. For RT-PCR, total cellular RNA, 2 μg from non-diabetic and diabetic rats were reverse-transcribed using an RNA PCR Kit, AMV (Takara, Kyoto, Japan). 5% of each reverse transcriptase product was amplified in the PCR reaction using the oligonucle-

Table 1. Total body weight and blood glucose level comparisons between diabetic (STZ) and control rats

Parameter	Group	2 weeks	4 weeks	3 months	6 months
Body weight (g)	Control	377.6±13.5	394.3± 2.0	614.8±17.1	637.7±22.2
	STZ	285.3± 2.5*	286.7±28.9*	304.5±16.9*	353.6±28.0*
Blood glucose level (mg/dl)	Control	95.5±11.0	96 ± 8.2	110 ± 3.3	85 ± 5.6
	STZ	279 ± 0.8*	330 ±55.5	304 ±15.9*	284.5±39.5*

Values shown are means±standard deviations. An asterisk ("*") indicates $p<0.05$ for the comparison between the control and diabetic rats.

otides described above. Polymerase chain reaction cycles were as follows: 95 °C, 3 min (once); 95 °C, 30 s; 55 °C, 1 min; and 72 °C, 45 s (25 cycles). RT-PCR products (about 372 bp) amplified with degenerate Ang-1 oligonucleotides from rat or RT-PCR products (about 453 bp) amplified with degenerate Ang-2 oligonucleotides from rat were then separated by 2% agarose gel electrophoresis. To investigate relative levels of Ang-1 and Ang-2 gene expression, semiquantitative analysis was then performed by measurement of the optical densities of the band with a fluorescence imager (FluorImager SI; Molecular Dynamics, Sunnyvale, CA) and its associated software WinRoof (Mitani Shoji, Fukui, Japan). The relative levels of mRNA expression were then calculated.

Real-time PCR: Total retinal RNA was extracted from 6 month old diabetic and control rats using RNAqueous-4PCR (Ambion, Austin, TX). First-strand cDNA was reverse transcribed from the total RNA using the First-Strand cDNA synthesis kit (Roche Pharmaceuticals, Mannheim, Germany) utilizing random hexamer nucleotides for initiation priming. Real-time PCR was performed using an ABI Prism 7700 PCR machine (Applied Biosystems, Foster City, CA). Primers and probes for rat VEGF was designed using Primer Express Soft-

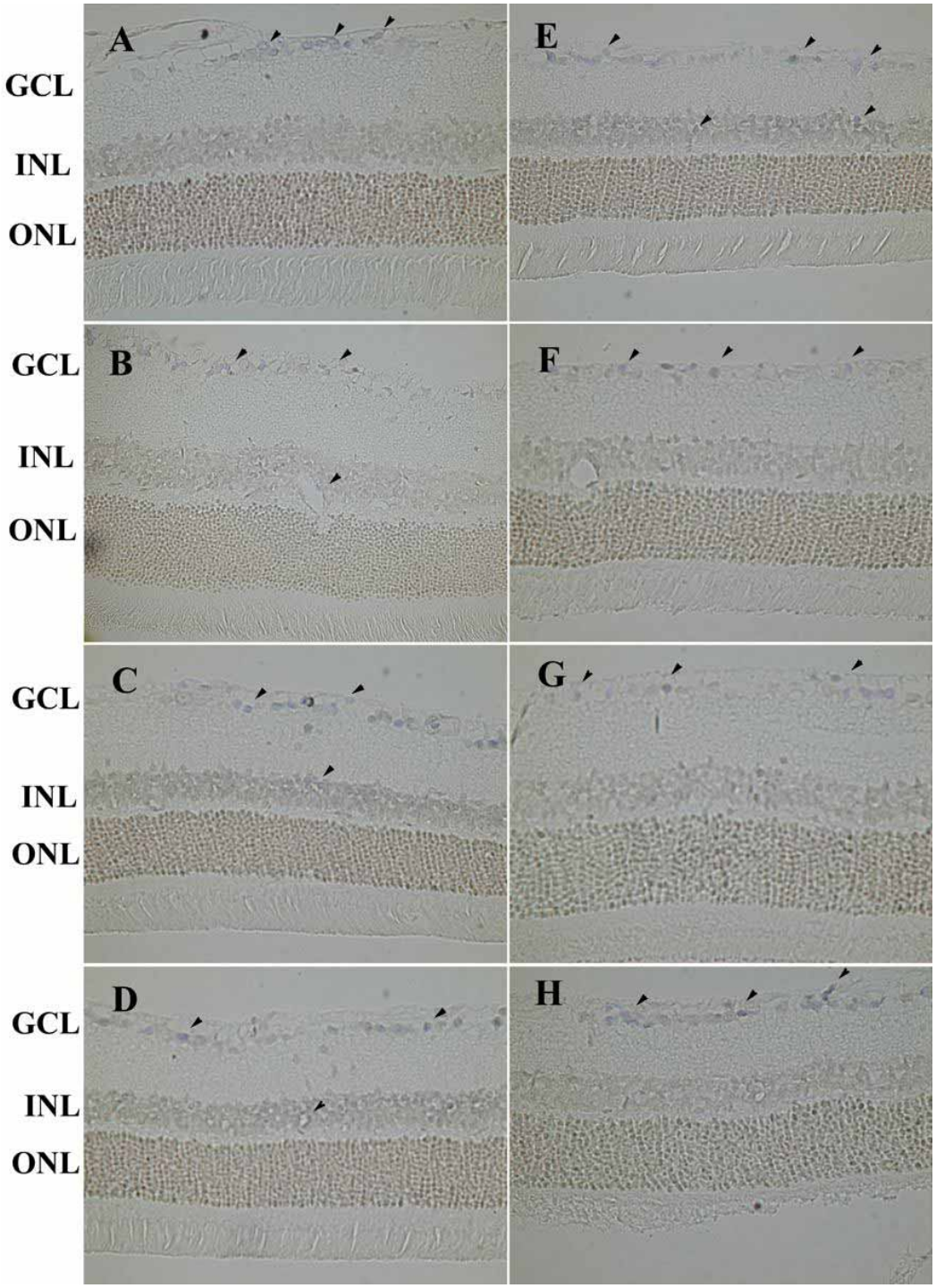


Figure 1. In situ hybridization analysis of Ang-1 during the development of diabetes. **A**-**D**: the retinas of STZ induced diabetic rats. **E**-**H**: the retinas of saline injected non-diabetic control rats. **A**,**E**: 2 weeks after injection. **B**,**F**: 1 month later after injection, **C**,**G**: 3 months after injection. **D**,**H**: 6 months later after injection. Weak Ang-1 mRNA expression was observed in the GCL and the INL (arrow heads). The intensity of Ang-1 mRNA expression was unchanged between diabetic and non-diabetic rats from 2 weeks to 6 months following STZ injection.

ware (version 2.0; Applied Biosystems). The real-time PCR cycle parameters were 48 °C for 30 min and 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min. The relative differences were determined using the CT method as outlined in the Applied Biosystems protocol. The starting mRNA copy number of the target sequence was established by determining the fractional PCR threshold cycle (CT) number at which a fluorescence signal generated during the replication process passed above a threshold value. The initial amount of target mRNA in each sample was estimated from the experimental CT value with a standard curve.

Western blot analysis: Detergent soluble lysates of retina were prepared as previously described [24]. Briefly, retinas were collected from both 6 months diabetic and age matched control rats and extracted separately with ice cold lysis buffer (50 mM Hepes, pH 7.4, 10 mM EDTA, 100 mM NaF (Sigma Chemical), 10 mM sodium pyrophosphate (Sigma Chemical), 1% Triton X-100, 10 mM Na_3VO_4 (Sigma Chemical), 20 μM leupeptin (Sigma Chemical), 1.5 μM aprotinin (Sigma Chemical), and 2 mM PMSF (Sigma Chemical)) for 30 min. Lysates were cleared by centrifugation at 12,000 rpm for 15 min at 4 °C, and supernatants were removed and diluted with an equal

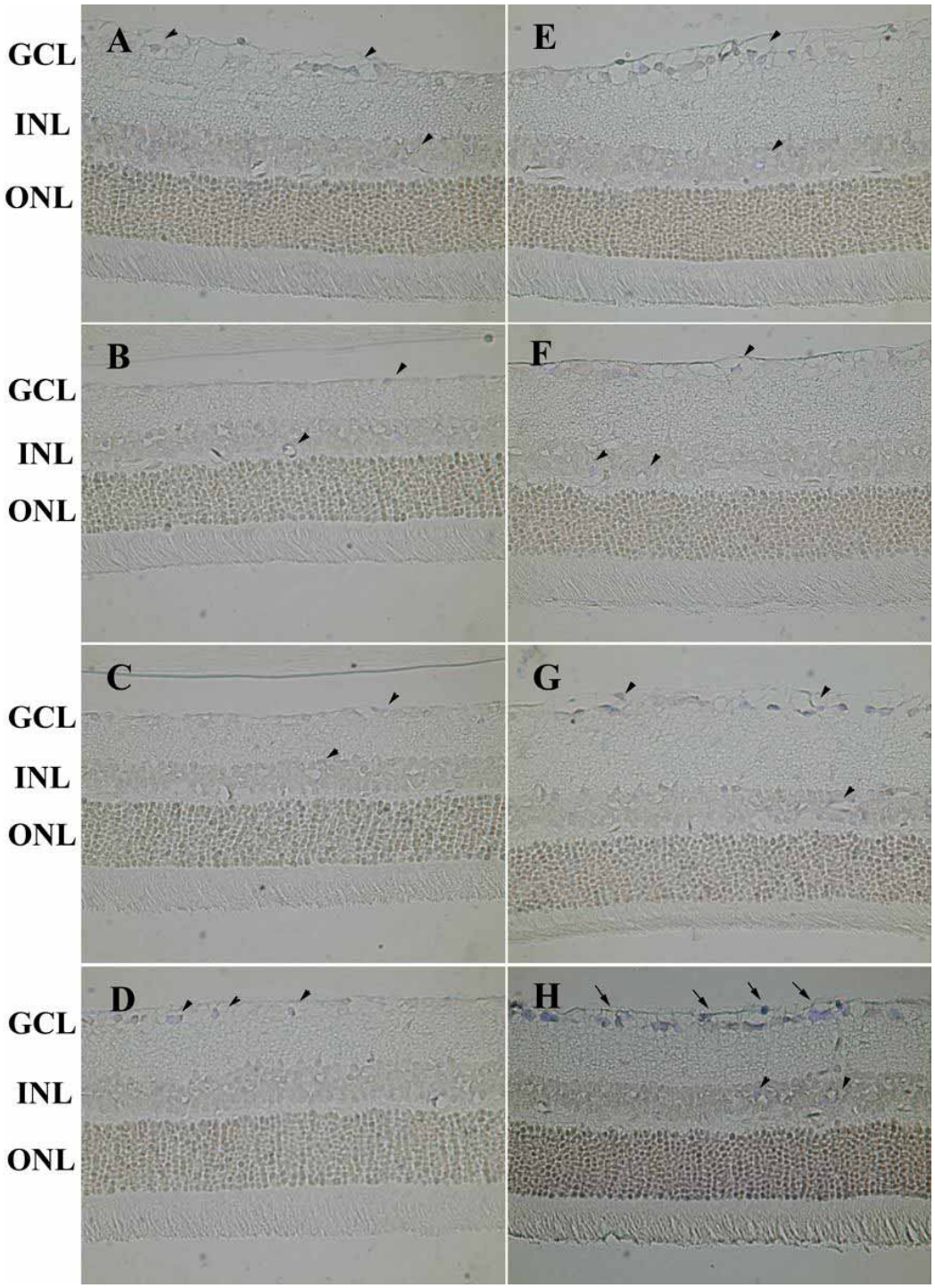


Figure 2. In situ hybridization analysis of Ang-2 during the development of diabetes. **A**-**D**: the retinas of STZ induced diabetic rats. **E**-**H**: the retinas of saline injected non-diabetic control rats. **A**,**E**: 2 weeks after injection. **B**,**F**: 1 month after injection, **C**,**G**: 3 months after injection. **D**,**H**: 6 months after injection. Weak Ang-2 mRNA expression was observed in the GCL and the INL at 2 weeks, 4 weeks and 3 months after STZ injection (arrow heads). The intensity of Ang-2 mRNA expression increased in the retina of diabetic rats at 6 months after STZ injection (arrows).

volume of 2% SDS sample buffer. Protein concentrations of the supernatants were determined with the bicinchoninic acid reagent (Pierce, Rockford, IL). Lysates were heated to 95 °C for 2 min, and equal volumes were subjected to SDS-PAGE under reducing conditions. To assay for Ang-1, Ang-2 and Tie-2, blots were incubated with polyclonal anti-Ang-1, Ang-2 and Tie-2 specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Lane loading was normalized by reblotting with a monoclonal anti-β-actin antibody (Sigma Chemical). Immunoblots were visualized using an enhanced chemiluminescence detection system (ECL, Amersham Biosciences, Piscataway, NJ) according to the instructions of the manufacturer.

Statistical analysis: Determinations for RT-PCR were performed in triplicate and results are expressed as means±standard error of the mean Student's t-test was used; 0.05 was selected as the α level.

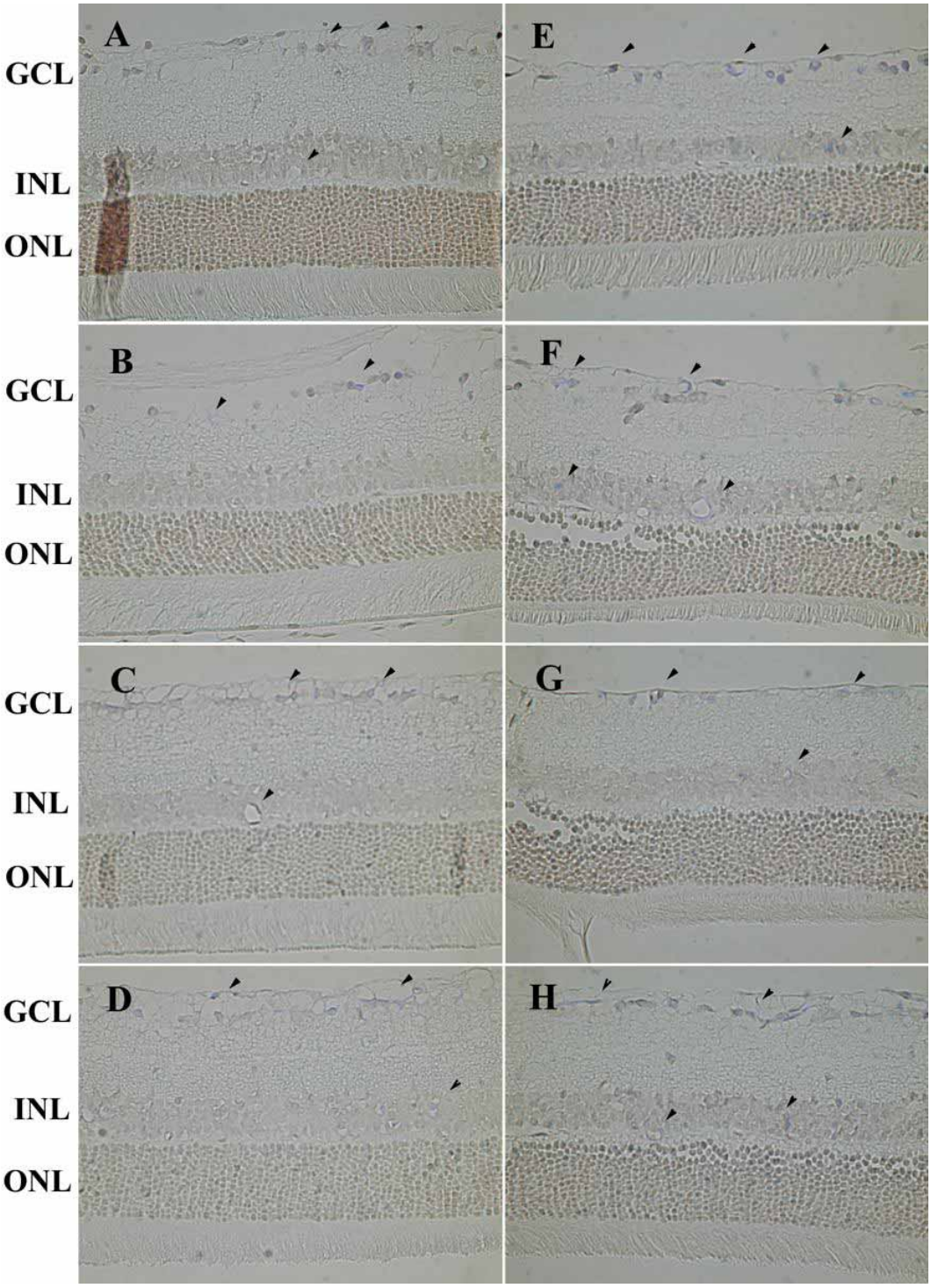


Figure 3. In situ hybridization analysis of Tie-2 during the development of diabetes. **A**-**D**: the retinas of STZ induced diabetic rats. **E**-**H**: the retinas of saline injected non-diabetic control rats. **A**,**E**: 2 weeks after injection. **B**,**F**: 1 month after injection, **C**,**G**: 3 months after injection. **D**,**H**: 6 months after injection. Tie-2 mRNA expression were observed in the GCL and the INL (arrow head). The intensity of Tie-2 mRNA expression was unchanged between diabetic and non-diabetic rats from 2 weeks to 6 months following STZ injection.

RESULTS

Expression of angiopoietins and the Tie-2 receptor in diabetic retinas: In situ hybridization was performed to analyze the expression of Ang-1, Ang-2, and Tie-2 in sections of eyes from STZ induced diabetic rats and then compare these patterns with those of non-diabetic control rats. An initial comparison between the body weight and plasma glucose levels of diabetic and control rats was also performed and is shown in Table 1. Diabetic rats had significantly lower body weights during the course of the experiment, 2 weeks to 6 months ($p<0.01$). Additionally, the plasma glucose levels of diabetic rats were significantly higher than those of the control rats ($p<0.01$). Ang-1 mRNA was expressed weakly in the ganglion cell layer (GCL) and the inner nuclear layer (INL; Figure 1). No remarkable difference was observed in Ang-1 expression between non-diabetic and STZ injected diabetic rats. Similarly, Ang-2 mRNA was also expressed weakly in the GCL and the INL (Figure 2). However, the expression level of Ang-2, although unchanged up to 3 months following STZ injection, increased at 6 months after STZ injection. Prominent Ang-2 expression was observed in the GCL of the diabetic rats at 6 months (Figure 2D). The Tie-2 receptor gene was also expressed in the GCL and the INL (Figure 3) but once again, no remarkable difference was observed between the expression of Tie-2 in non-diabetic and diabetic rats.

Increase of Ang-2 mRNA expression in the diabetic retina: To examine the relative expression levels of Ang mRNA during long term diabetes, semiquantitative RT-PCR experiments were performed using β-actin for normalization (Figure 4). Amplified PCR bands using both Ang-1 (372 bp) and Ang-2 primers (453 bp) were obtained from cDNA derived from the retinas of non-diabetic and diabetic rats six months after STZ injection. Ang-1 mRNA expression levels in diabetic retinas were equivalent to the non-diabetic control (Figure 4A, top). The mean±standard error of the mean expression in diabetic rats was 116±6% of that in non-diabetic controls (Figure 4B, top). In contrast to Ang-1, retinal expression of Ang-2 increased to 181±6% of controls 6 months after STZ injection (Figure 4A and Figure 4B, bottom).

Increase of Ang-2 protein expression in the diabetic retina: To elucidate expression levels of Ang-1, Ang-2 and Tie-2 protein in diabetic retinas, western blot analyses were performed. Consistent with the results of both in situ hybridization and RT-PCR analyses, Ang-2 protein expression was significantly ($p=0.039$) upregulated in retinas from diabetic rats compared to that from age matched non-diabetic control rats after 6 months. In contrast, both Ang-1 and Tie-2 protein levels were similar between diabetic and non-diabetic control rats at this time point (Ang-1, $p=0.1489$; Tie-2, $p=0.3865$; Figure 5A,B).

Increase of VEGF mRNA expression in the diabetic retina: We previously reported that VEGF, a central inducer of angiogenesis and whose expression is also reported to be increased in the diabetic retinas [25,26], can upregulate Ang-2 expression in endothelial cells [20]. To explore whether VEGF could be the inducer of Ang-2 expression in the diabetic retinas, we analyzed VEGF mRNA expression level in the diabetic retinas by real time PCR. VEGF mRNA expression was significantly ($p=0.0495$) upregulated in retinas from diabetic rats compared to that from age matched non-diabetic control rats after 6 months. The mean±standard error of the mean expression in diabetic rats was 233±44% of that in non-diabetic controls (Figure 5C).

Overexpression of Ang-2 is related to pericyte loss: To investigate changes in pericytes concurrent with Ang-2 overexpression, a double staining experiment using in situ

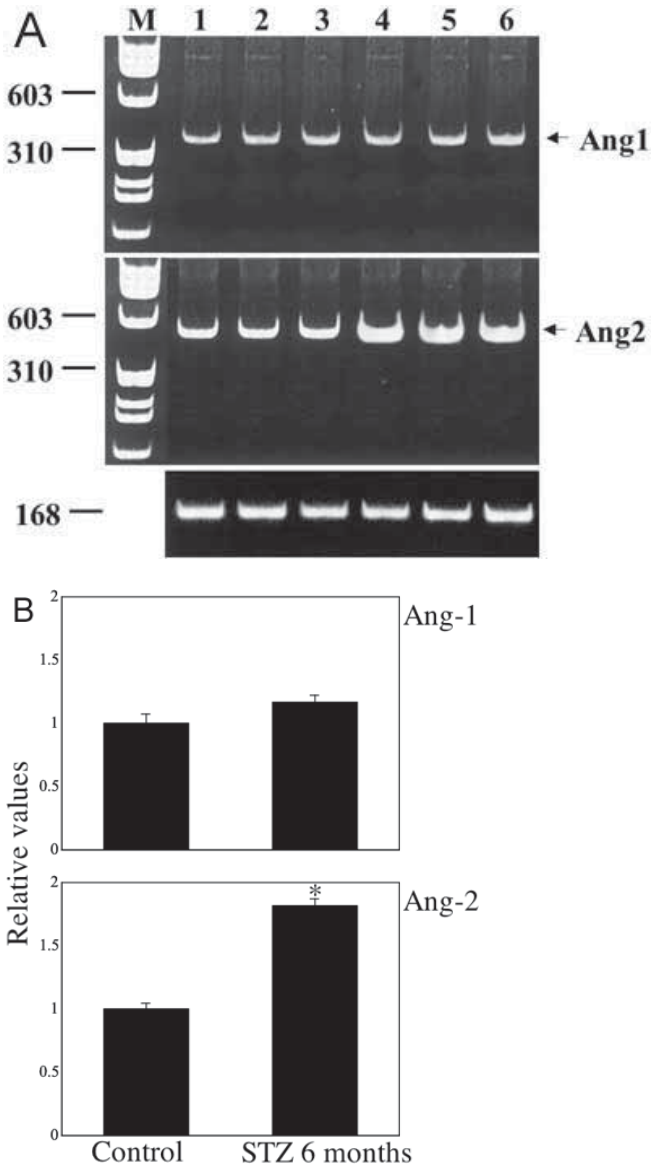


Figure 4. RT-PCR experiments to determine Ang-1 and Ang-2 gene expression in 6 months diabetic and control retinas. PCR was performed using (**A**, top) Ang-1 primers and (**A**, middle) Ang-2 primers after cDNA concentration was normalized to (**A**, bottom) β-actin gene expression. PCR products of expected lengths (Ang-1: 372 bp and Ang-2: 453 bp) were obtained (arrow). Lane 1-3: non-diabetic control rats; lane 4-6; diabetic rats; Lane M: X174/HaeIII marker. **B**: Relative intensities of PCR bands (top: Ang-1, bottom: Ang-2). In contrast to Ang-1, retinal expression of Ang-2 increased 6 months after STZ injection. An asterisk ("*") indicates $p<0.0005$ compared to control.

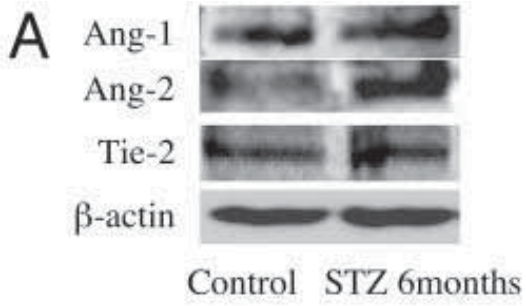
hybridization and immunohistochemistry was performed. Initially, retinas were stained by in situ hybridization using an Ang-2 probe and at 6 months following STZ injection, prominent Ang-2 expression was observed in the GCL. The same slides were used for immunohistochemical staining using an anti-αSMA antibody as a marker for periendothelial cells. In retinas of non-diabetic control rats, Ang-2 mRNA expression was observed only very weakly in the GCL and INL, while αSMA positive cells were observed in the GCL and the INL (Figure 6, left). In retinas of diabetic rats, Ang-2 mRNA expression was observed prominently in the GCL and INL, but αSMA positive pericytes decreased in both these regions compared to non-diabetic controls (Figure 6, right). Furthermore, this decrease of αSMA positive cells was especially prominent in areas alongside Ang-2 expressing cells.

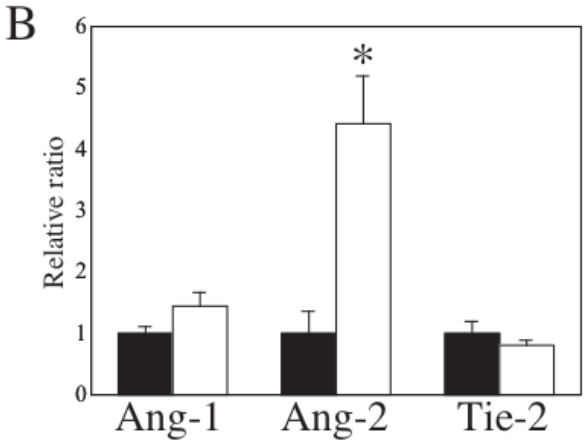
DISCUSSION

Interaction between endothelial cells and pericytes has been shown to be a key regulatory mechanism for the functional properties of endothelial cells. The contact induced inhibitory effect of pericytes on the proangiogenic activity of endothelial cells is dependent, at least in part, on plasmin mediated activation of the latent form of TGF-β, which is produced by both pericytes and endothelial cells [27]. Based on the findings from Ang-1 knockout and Ang-2 transgenic mice, Ang-2 is suggested to play a role in suppressing such pericyte-endothelial cell interactions [28,29]. Indeed, Ang-2 expression is prominently upregulated in neovascular vessels where peri-endothelial cells are degenerative [30]. Therefore, we hypothesized that Ang-2 may be causally linked to pericyte loss in diabetic retinopathy. In the study described herein, we first demonstrated that Ang-2 is upregulated in the retina of diabetic rats whereas Ang-1 and Tie-2 are relatively stable. Ang-2 upregulation was observed in the GCL and the INL and with co-staining of SMA, we found that this upregulation correlated with depletion of peri-endothelial cells. These data suggest that an increase in Ang-2 might have possible effects on pericyte loss in diabetic retinas.

In adults, Ang-1 is constitutively expressed but regulation of this expression was demonstrated only during tumorigenesis [31,32] and in the ovulatory cycle [14,33]. The regulation of the Ang-1 gene in the retinas of diabetic animals has not been previously reported. In the present study, we have demonstrated using in situ hybridization that Ang-1 mRNA is expressed weakly in the GCL and the INL. The Tie-2 gene was also expressed in the GCL and the INL and it has been shown that in adult organs, constitutive expression of Ang-1 with concomitant phosphorylation of Tie-2 receptor tyrosine residues suggests that Tie-2 activity is important for the maintenance of a quiescent mature vasculature [34].

We also found no remarkable alterations in Ang-1 and Tie-2 expression between non-diabetic rats and STZ injected diabetic rats during the 6 months period that we performed our experiments. Ang-1 has been shown to inhibit diabetes induced leukocyte adhesion and subsequent endothelial damage [35]. Ang-1 is also reported to suppress diabetes induced





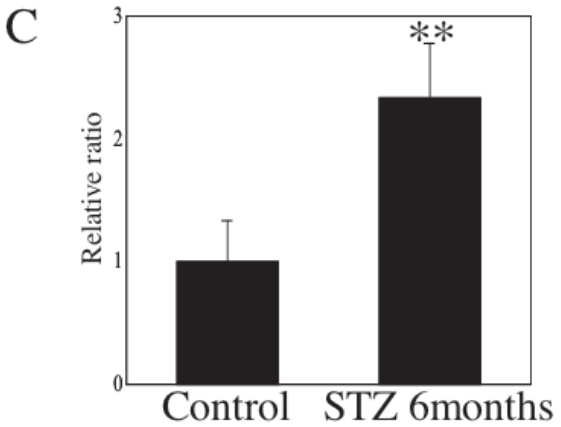


Figure 5. Western blot analysis to determine Ang-1, Ang-2 and Tie-2 protein expression in 6 months diabetic and control retinas. Lane loading was normalized by reblotting with a monoclonal anti-β-actin antibody. **A**: Representative bands of western blot analyses. **B**: Relative expression levels of Ang-1, Ang-2 and Tie-2 protein (n=4). Black columns; diabetic retina at 6 months after STZ induction, White columns; age matched non-diabetic retina. An asterisk ("*") indicates $p<0.05$ compared to Ang-2 protein expression level of non-diabetic retina. In contrast to Ang-1 and Tie-2, Ang-2 protein level was selectively upregulated 6 months after STZ injection. **C**: Real-time PCR analyses of retinal mRNA expression of VEGF in the diabetic and non-diabetic control retinas (n=4). Double asterisks ("**") indicate $p<0.05$ compared to VEGF mRNA expression level of non-diabetic retina. VEGF mRNA was significantly upregulated in 6 months diabetic retinas.

increases in retinal vasculature leakage [35]. These data suggest that it is not likely that alterations in expression of these genes are the primary mechanisms of diabetes induced pathologies. To know if Ang-1 is changed earlier, further time course experiments will be necessary.

Similar to Ang-1, Ang-2 mRNA was expressed only weakly in the GCL and the INL, the localization of which is consistent with the report of Hackett et al [29]. In their study, using the Ang-2 heterozygous mouse, Ang-2 expressed by neural cells is associated with the changes of retinal oxygen supplies and vascular remodeling in GCL and INL. In the present study, we have demonstrated for the first time that Ang-2 mRNA expression is upregulated in diabetic retinas. Following in situ hybridization experiments, Ang-2 expression was unchanged from 2 weeks to 3 months after STZ injection but was increased at 6 months. Prominent Ang-2 expression was observed in the GCL and the increase in the retinal expression levels of Ang-2 were confirmed by both RT-PCR and western blot analyses. The localization of Ang-2 suggests that Ang-2 might have effects on neural cells and indirect effects on vascular cells in the diabetic retinas [36]. Additionally, since TaqMan PCR would give a more detailed data than RT-PCR, further evaluation of expression levels of these molecules using TaqMan PCR are required in a future study.

At an early stage of tumorigenesis, Ang-2 has been shown to be induced in tumor microvessels, resulting in the disruption of the endothelial cell-pericyte interaction, endothelial apoptosis, and vessel regression [37,38]. In glioblastoma histology, Ang-2 has also been shown to be locally upregulated in the area of the disruption of endothelial and pericyte interaction [31]. In the present study, we also observed disruption of endothelial cell-pericyte interaction in the areas where Ang-2 is upregulated. These findings might suggest that selective Ang-2 increase might be a possible pathway for pericyte degeneration and loss or the neuronal retinal changes observed in diabetes. However, in our study, there is not sufficient data about the causal relationship between Ang-2 increase and retinal pericyte loss. Pericytes might be dying in spite of Ang-2 upregulation as well as because of it in the diabetic retinas.

Ang-2 has been reported to act differently in the absence and presence of VEGF. In the presence of VEGF, Ang-2 plays a proangiogenic role. By contrast, Ang-2 promotes endothelial cell death and vessel regression in the absence of VEGF [39,40]. Additionally, retinal VEGF mRNA was shown to be upregulated by experimental diabetes [25,26] and VEGF has been reported to upregulate Ang-2 expression in retinal endothelial cells [20]. Consistent with these reports, VEGF mRNA was significantly upregulated in 6 months diabetic retinas in the present study. These results suggest that VEGF in-

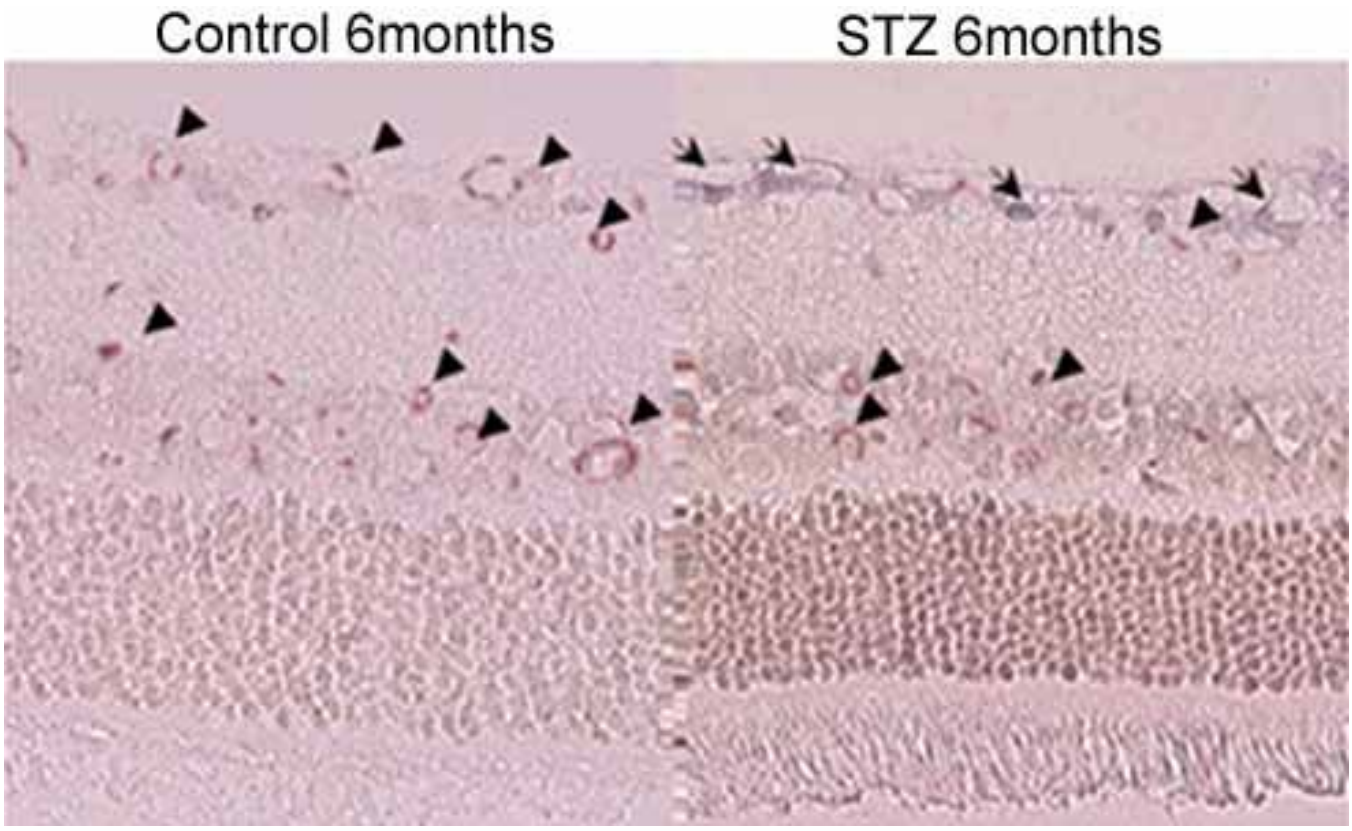


Figure 6. In situ hybridization analysis of Ang-2 expression followed by co-staining with anti-αSMA antibody. Control and 6 months diabetic animals were compared. Ang-2 mRNA expression were observed in the GCL and the INL (arrow head) in control animals and was increased in these layer by diabetes. αSMA positive cells were stained in the GCL and the INL (arrow). In diabetic rats, αSMA positive staining decreased compared to non-diabetic rats specifically in regions surrounding Ang-2 overexpressing cells.

duced upregulation of Ang-2 might stimulate pericyte loss and play proangiogenic roles in diabetic retinopathy. In contrast, recombinant Ang-1 had been reported to rescue retinal disorders induced by the absence of perivascular mural cells [41]. These studies suggest that the local treatment of recombinant Ang-1 might be a new therapeutic way to stabilize vessels by protecting the dropout of perivascular cells and that, in contrast, administration of recombinant Ang-2 in the presence of abundant VEGF might exacerbate diabetic retinopathy by promoting neovascularization. To fully delineate the causal relationship between Ang and pericyte loss in the diabetic retinas, further experiments such as administration of Ang-1 or Ang 2 and Ang over expressing transgenic animals will be necessary in the future study.

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