Role of the Polyol Pathway in High Glucose–Induced Apoptosis of Retinal Pericytes and Proliferation of Endothelial Cells

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PURPOSE. The selective degeneration of pericytes and the proliferation of endothelial cells (ECs) appear to be associated with microaneurysm formation, an initial deficit in the early stage of diabetic retinopathy. The preventive effect of aldose reductase (AR) inhibitor (ARI) for high-glucose–induced pericyte loss and EC growth was investigated.

METHODS. The effect of high glucose (30 mM) exposure in the presence or absence of ARI for the cell growth of porcine pericytes and ECs was examined with the use of an in vitro coculture system to mimic the interaction between pericytes and ECs. To determine the role of transforming growth factor (TGF)-β, its amount in culture media was measured, and the effects of the treatment of TGF-β or neutralizing antibody on EC growth were examined.

RESULTS. Abundant expression of AR and increased levels of polyol and apoptosis induced by high glucose were observed in pericytes, but not in ECs. ECs overexpressing AR cultured in high-glucose medium showed decreased cell viability. The growth-inhibitory effect of ECs on coculture with pericytes was attenuated by exposure to a high glucose concentration. Biochemical assays disclosed that the levels of active TGF-β in media were linked to EC growth. Supply of active TGF-β to coculture medium containing 30 mM D-glucose restored the inhibitory activity on EC growth.

CONCLUSIONS. ARI rescued pericytes from high-glucose–induced apoptosis and maintained the levels of TGF-β, resulting in the prevention of cocultured EC growth. (Invest Ophthalmol Vis Sci. 2008;49:3216–3225) DOI:10.1167/iovs.07-10435

Diabetic retinopathy is the main cause of visual loss and is considered to result from alteration of capillary hemodynamics. It is recognized that pericyte loss is a typical sign of early microangiopathy of the diabetic retina. Microaneurysms are accompanied by endothelial hyperplasia resulting from aberrant proliferation, basement membrane thickening, and decreased number of pericytes. Pericyte loss has been reported to occur in diabetic and galactose-fed dogs, which show changes in retinal vessels similar to those seen in human diabetic retinopathy such as microaneurysms, hemorrhages, and nonperfused areas. Experimental evidence suggests that these retinal changes can be prevented by aldose reductase inhibitor (ARI). Excess sugar alcohol (polyol) accumulation, catalyzed by the enzyme aldose reductase (AR) during hyperglycemia, has been implicated in the pathogenesis of diabetic complications including retinopathy.

Numerous studies have shown that increased AR activity leads to multiple metabolic consequences including, but not limited to, nonenzymatic glycation, activation of protein kinase C, oxidative stress, and poly(ADP-ribose) polymerase (PARP) activation. Inhibitors of these factors, which do not affect retinal polyl accumulation, have the potential to prevent diabetes-induced abnormality, including high-glucose–induced apoptosis of pericytes, formation of acellular capillaries, and vascular endothelial growth factor (VEGF) production. Oxidative stress, PARP activation, and overexpression of VEGF could be counteracted by the treatment of ARI. Therefore, it is probable that these multiple metabolic mechanisms worked together and contributed to the preventive effect with ARI.

Immunohistochemical studies have demonstrated that abundant expression of AR is observed in pericytes isolated by trypsin digestion from human and dog retina. Apoptotic cell death is induced in pericytes cultured in high-glucose medium and can be prevented by treatment with ARI suggesting that the polyol pathway through AR plays an important role in the pathogenesis of pericyte loss. Apoptosis has not been found in galactose-exposed retinal dog endothelial cells (ECs) that have low AR content and activity, and clearly manifested apoptotic changes have been identified in high-glucose–exposed bovine ECs and retinal ECs of rats and human subjects with diabetes. It is suggested that PARP activation is involved in the diabetes-induced death of rat ECs through the regulation of nuclear factor kappa B. Moreover, Dagher et al. showed that AR protein is detectable in rat and human ECs. Therefore, the role of the polyol pathway in the abnormal dynamics of ECs under hyperglycemia remains unclear.

In vitro coculture systems have shown that pericytes have a capacity to inhibit EC growth under normal glucose conditions. It has also been demonstrated that this EC growth inhibition on coculture with pericytes is mediated by the activated form of transforming growth factor β (TGF-β). TGF-β, a member of a family of 25-kDa homodimeric polypeptides, has potent inhibitory activities on cell growth. TGF-β is usually secreted as an inactive latent form. Under single culture conditions, pericytes or ECs secrete only latent TGF-β; however, in coculture, the latent TGF-β is converted to the activated form. In the present study, we used a coculture system to investigate whether the apoptosis of pericytes induced by exposure to a high glucose concentration affects the growth of ECs and, if so, whether the uncontrolled EC growth may be mediated by altering the level of bioactive TGF-β. The effect of an ARI, fidarestat, on the EC growth associated with glucose-induced apoptosis of pericytes was also examined.

METHODS

Cell Culture and Transfection

Capillary ECs and pericytes were isolated separately from porcine retinal microvessels, as described previously. ECs were maintained...
on fibronectin-coated culture dishes in Dulbecco modified Eagle medium (DMEM; Gibco, Grand Island, NY) with 10% horse serum (Sigma, St. Louis, MO), endothelial cell growth supplement (ECGS; Sigma), and heparin sodium salt at 50 μg/mL. Pericytes were maintained in DMEM with 15% fetal bovine serum (FBS). Cells were identified as ECs, and pericytes were identified by immunohistologic staining with antisera against von Willebrand factor and muscle-type actin, respectively.31 Culture media containing 0.2% bovine serum albumin (BSA) and no added growth factors or serum were used in single- and coculture studies. Although cells were routinely propagated with growth supplement and serum, they also grew at a lower doubling rate in the absence of growth factor (media containing 0.2% BSA). A construct containing an enhanced green fluorescent protein (EGFP) and AR cDNA was generated with the “living color system” (Clontech, Mountain View, CA) for eukaryotic expression. Total RNA was isolated using the single-step guanidine thiocyanate/phenol/chloroform extraction method (Trizol Regent; Invitrogen, Carlsbad, CA) from porcine retina and was converted to cDNA (Superscript II Reverse Transcrtipase; Invitrogen). Amplified product by polymerase chain reaction coding the full length of porcine AR was cloned into the plasmid vector pEGFP-c1 using the appropriate restriction enzymes. Transfection was performed by lipofectamine (Invitrogen) according to the manufacturer’s protocol. To establish AR overexpression, ECs transfected with the EGFP-AR construct were exposed to the medium containing anti-biotic (Geneticin; Invitrogen, Carlsbad, CA) at 500 μg/mL for up to 21 days.

Coculture Studies

ECs were cocultured with pericytes as described previously.24 Cell division of pericytes was arrested by treatment with mitomycin C (10 μg/mL; Sigma) for 2 hours. Growth-arrested pericytes were rinsed with PBS, removed using 0.05% trypsin, and plated into 24-well culture dishes coated with fibronectin. After overnight attachment, the number of pericytes was determined, and an equal number of ECs was plated (day 0). On the following day (day 1), the culture wells were randomly divided into three groups. One group was cultured in the same DMEM medium (5 mM glucose), the second group was cultured in DMEM containing 30 mM glucose, and the third group received DMEM containing 30 mM α-glucose and 10 mM fructose ((2S,4S)-6-fluoro-2′,5′-dioxospiro (chroman-4,4′-imidazolidine)-2-carboxamide; Sanwa Kagaku Kenkyusho, Nagoya Japan) as an ARI. Pericytes were distinguished from ECs by their size, morphologic difference, and lack of Hoechst nuclear staining, as described. Cell counting and calculation were automatically performed (Mac-SCOPE; Mitani Corporation, Tokyo, Japan).

To determine the effect of neutralization of TGF-β, anti-TGF-β IgG (R&D Systems, Minneapolis, MN) was added at 150 μg/mL after overnight attachment of ECs. Controls consisted of cocultures that received an equivalent volume of normal rabbit serum. To examine the effect of TGF-β on cocultures, ECs were plated on pericytes, and 24 hours later the cells were washed twice with DMEM without serum and treated with TGF-β1 (R&D Systems) at 1 ng/mL in 5 mM or 30 mM α-glucose with 0.2% BSA.

Immunohistochemical Cell Staining

To test the endogenous expression, immunoperoxidase cell staining was performed (ABC Staining System; Santa Cruz Biotechnology, Santa Cruz, CA). Pericytes and ECs were fixed in ~10°C methanol for 5 minutes, washed with PBS, and incubated in 0.5% H2O2 in PBS for 5 minutes to quench endogenous peroxidase activity. The cells were washed in PBS twice for 5 minutes and incubated for 1 hour in 1.5% blocking goat serum. After washing, the cells were incubated with anti-AR or anti-von Willebrand factor–specific antibody (Santa Cruz), washed three times, and incubated with biotinylated secondary antibody for 30 minutes after three washes, the sections were incubated with avidin and biotinylated horseradish peroxidase enzyme reagents for 30 minutes, and then incubated in DAB with substrate buffer for 5 minutes.

Western Blot Analysis

Pericytes or ECs were washed, harvested, and lysed in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS). Equal amounts of protein were subjected to 10% to 20% gradient SDS-PAGE and then blotted onto polyvinylidene difluoride membranes (Immobilon P; Millipore, Billerica, MA). Blocking was performed with 5% nonfat dry milk in 0.1% Tween 20 in PBS, and then the membranes were incubated overnight at 4°C with anti-AR antibody at 1:1000 dilution. After four washings with PBS-T, the membranes were incubated with horseradish peroxidase–labeled anti–goat IgG. The specific band was visualized with luminol reagent (Santa Cruz Biotechnology). The membranes were then stripped and reprobed with anti–β-actin antibody (Santa Cruz Biotechnology) or anti–von Willebrand factor antibody. The density of each band was analyzed with a luminescent image analyzer (LAS-1000 Plus; Fuji Film, Tokyo, Japan).

TUNEL Staining and Viability Assay

To detect apoptosis, the terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) assay was carried out using a fluorescein detection kit (In Situ Cell Death Detection Kit, Fluorescein; Roche, Indianapolis, IN). In brief, pericytes/ECs were incubated in DMEM containing 5 mM or 30 mM glucose for 9 days, then fixed with freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) for 30 minutes. After a rinse with PBS, 50 μL reaction mixture was added, and the samples were incubated at 37°C for 1 hour. After washing with PBS three times, the samples were directly analyzed using a fluorescence microscope.

The nuclei were stained with the vital nuclear dye bisbenzamide (Hoechst 33342). To examine cell viability, the colorimetric MTS assay (Promega, Madison, WI) was performed in accordance with the manufacturer’s protocol. Absorbance at 490 nm was measured after 4 hours with an ELISA reader.

Determination of Polyol Content

Pericytes/ECs were cultured with DMEM containing 5 mM or 30 mM α-glucose. After incubation for 9 days, the cells were washed with DMEM, and the supernatant was removed by centrifugation. The sorbitol content was measured by liquid chromatography–tandem mass spectrometry (LC/ MS/MS) using a modification of the Guerrant method.32

Detection of Biologically Active TGF-β

Bioactive TGF-β1 in the culture supernatant was determined (TGF-β1 ImmunoAssay system; Promega). Briefly, 96-well plates were coated with TGF-β coat polyclonal antibody, which binds specifically to soluble TGF-β1. After washing, the amount of specifically bound polyclonal antibody was measured with an antibody conjugated to HRP by reading the absorbance at 450 nm. To assay for total TGF-β, an acid treatment procedure to activate latent TGF-β was performed in accordance with the manufacturer’s protocol.33

Statistical Analyses

Statistical analyses for multiple comparisons of mean values between cell preparations were performed by one-way analysis of variance, followed by the Fisher exact test. All experiments reported were performed at least four times.

RESULTS

Polyol Accumulation and Apoptosis Induced by High Glucose in Pericytes but Not in ECs

To investigate the role of AR in pericyte loss, we initially examined the levels of AR expression in porcine retinal peri-
cytes and ECs. Immunohistologic staining showed that abundant AR protein was located in the cytoplasm of pericytes, whereas less intense staining was observed in ECs (Fig. 1A). Protein blot analysis was conducted to estimate the level of AR expression in pericytes and ECs. As shown in Figures 1B and 1C, the level of AR expression in pericytes was approximately 15-fold higher than in ECs. Thus, both methods revealed markedly higher levels of AR expression in pericytes than in ECs.

Next, to confirm the physiological role of AR, pericytes and ECs were separately exposed for 9 days to a high concentration (30 mM) of D-glucose in the presence or absence of ARI. TUNEL staining revealed that exposure to high glucose induced apoptotic cell death in pericytes but not in ECs (Figs. 2Aa, 2B). The nuclei of TUNEL-positive cells showed the typical dense and fragmented appearance of apoptosis that was also detected by Hoechst nuclear staining (Fig. 2Bb). The percentage of apoptotic cells among pericytes resulting from high-glucose exposure was significantly reduced by ARI treatment ($P < 0.05$; Fig. 2Ba, lane 4).

To investigate whether the induction of apoptosis is associated with polyol accumulation, the amount of intracellular sorbitol was measured. The polyol level in pericytes cultured with 30 mM D-glucose was approximately sixfold higher than that in pericytes cultured with 5 mM D-glucose (Fig. 2Ca), whereas no significant increase of polyol accumulation was observed in ECs (Fig. 2Cb). Treatment with ARI had no effect on polyol levels in the presence of 5 mM D-glucose but almost normalized polyol levels in the presence of 30 mM D-glucose (Fig. 2Ca). Exposure to 5 mM D-glucose + 25 mM L-glucose, which cannot be metabolized, hardly affected apoptosis induction or polyol accumulation in pericytes and ECs.

**Attenuated Viability of ECs Overexpressing AR Induced by High Glucose Exposure**

To determine whether the difference in physiological response between pericytes and ECs depends on the level of AR expression, we investigated the effect of AR overexpression in ECs exposed to a high glucose concentration. Protein blot analysis was conducted to validate the expression of EGFP-AR in ECs. A 60-kDa band of EGFP-AR protein was detectable only in the EGFP-AR-transfected ECs. We then tested whether the overexpression of AR affected the activity of the polyol pathway by measuring sorbitol concentrations in EGFP-AR-transfected ECs (Fig. 3B). Intracellular sorbitol concentrations of ECs cultured with 30 mM D-glucose were approximately fourfold higher than those of ECs cultured with 5 mM D-glucose. This increase in the amount of sorbitol was significantly inhibited by treatment with ARI.

To determine the effect of glucose on the survival of ECs overexpressing AR, MTS assay was performed. When EGFP-AR-overexpressing ECs were cultured in 30 mM D-glucose, cell viability was significantly decreased, as occurred with pericytes that had been transfected with the EGFP vector (Fig. 3C). Treatment with ARI was able to restore cell viability to the control level.

**Effect of High Glucose on Growth Inhibition of ECs Cocultured with Pericytes**

To clarify the influence of pericyte dropout on EC growth under high glucose conditions, a coculture system was used. The pretreatment of mitomycin C inhibited the growth of pericytes (compare Figs. 4A and 4B) and ECs (compare Figs. 4D and 4E) at least up to 9 days, indicating the inhibition of mitotic activity. The number of monocultured ECs was continuously increased (Fig. 4D); however, the growth of ECs cocultured with pericytes with 5 mM glucose was significantly inhibited (compare Figs. 4D and 4F). When pericytes were monocultured with 30 mM D-glucose, their number gradually decreased (Figs. 4A, 4B), but EC growth was not affected (Figs. 4D, 4E). When ECs were cocultured with pericytes in medium containing 30 mM D-glucose, EC growth was inhibited for 6 days, but thereafter growth was rapid (Fig. 4F). There was an insignificant difference between the numbers of ECs monocultured and cocultured with 30 mM D-glucose at 9 days (compare Figs. 4D and 4F).

In monocultures, treatment with ARI suppressed the decrease of pericyte number because of high glucose exposure (Figs. 4A, 4B), whereas EC growth was unaffected (Figs. 4D, 4E). On coculture with 30 mM D-glucose in the presence of ARI, pericyte loss was prevented (Fig. 4C) and EC growth was inhibited throughout the culture period (Fig. 4F).

**Decrease in the Level of Bioactive TGF-β on Coculture Mediated by High Glucose Exposure**

It has been reported that the activation of TGF-β by coculture mediates EC growth. Therefore, we hypothesized that the level of active TGF-β would be influenced by exposure to high glucose and, thus, would affect EC growth. We measured the amount of bioactive TGF-β by ELISA assay (Emax; Molecular Devices, Sunnyvale, CA; Fig. 5). The culture supernatant of pericytes (Fig. 5A) or ECs (Fig. 5B) contained low levels of bioactive TGF-β, whereas that of media obtained from cells cocultured in 5 mM D-glucose was markedly high (Fig. 5C). The high amount of bioactive TGF-β was dramatically decreased on exposure of cocultures to a high (30 mM) glucose concentration (Fig. 5C). This reduction of TGF-β was strongly prevented by treatment with ARI (Fig. 5C). We then investigated whether any latent TGF-β was present in medium conditioned by coculture by subjecting the medium to acid activation to convert any latent TGF-β to the active form (Fig. 5D). The level of active TGF-β in cocultured media containing 30 mM D-glucose was found to be enhanced, indicating the presence of the latent form of TGF-β. On the other hand, the levels of bioactive TGF-β in cocultures containing 5 mM or 30 mM D-glucose and treated with ARI remained unchanged by acid activation, indicating that the TGF-β already present was fully activated.

**Effect of Supply of Activated TGF-β on EC Growth Cocultured with Pericytes**

To clarify whether active TGF-β actually inhibits EC growth, neutralizing antibodies against TGF-β were added to EC-pericyte cocultures after EC attachment, and the cell number was counted on day 9 of culture (Fig. 6). In the presence of neutralizing antibodies, the inhibitory effect of EC growth by cocultures was counteracted by 5 mM D-glucose (Fig. 6A, compare lanes 2 and 3). The addition of normal rabbit serum had no effect on the number of ECs (data not shown). In the presence of 30 mM D-glucose, the addition of anti–TGF-β IgG had no effect on the growth of cocultured ECs, indicating the absence or low level of active TGF-β (Fig. 6B, compare lanes 2 and 3).

EC-pericyte cocultures with 30 mM glucose did not maintain a high level of bioactive TGF-β, as shown in Figure 5. Therefore, we next determined whether adding bioactive TGF-β could rescue the ECs from growth inhibition (Fig. 6). The number of ECs after treatment with TGF-β (1 ng/mL) was significantly lower than after coculture with 30 mM D-glucose, suggesting an effect of TGF-β on EC growth (Fig. 6B, compare lanes 2 and 4).

**DISCUSSION**

Orlidge et al. showed that the growth inhibition of ECs on coculture with pericytes resulted from the activation of TGF-β.
This finding raised the possibility that pericyte degeneration may attenuate TGF-β activity, thus enhancing EC growth. In the present study, we demonstrated that ARI treatment was able to prevent the apoptosis of pericytes induced by a high glucose concentration and that this rescue from pericyte loss was essential for maintaining the level of bioactive TGF-β and controlling EC growth.

As observed previously for humans and dogs, high AR expression was observed in capillary pericytes isolated from porcine retina but not in ECs. Polyol accumulation and apo-
ptosis induction occurred in pericytes but not in ECs cultured in high-glucose medium. Based on these results, it is likely that the selective degeneration of pericytes is linked to AR activity. Indeed, the induction of pericyte apoptosis was completely inhibited by ARI. Moreover, AR-overexpressing ECs showed decreased cell viability and polyol accumulation, as did pericytes, suggesting that the physiological difference in response to high-glucose exposure is attributable to the level of AR expression and is not a cell-specific feature of pericytes and ECs. It was noteworthy that the overexpression of AR was not

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**FIGURE 3.** (A) Protein blot analysis of cellular extracts of ECs expressing EGFP-AR (lane 2) and EGFP-vector-transfected pericytes (lane 1) and ECs (lane 3) immunostained with anti-AR antibody. (B) Determination of polyol concentrations in EGFP-AR-transfected ECs cultured with 5 or 30 mM d-glucose in the presence or absence of 10 µM ARI. (C) Cell viability assessed by MTS assay of EGFP-vector-transfected ECs and pericytes and EGFP-AR-transfected ECs. After transfection, cells were incubated with 5 or 30 mM d-glucose in the presence or absence of 10 µM ARI. Values are mean ± SE of five different experiments.

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**FIGURE 4.** Growth of pericytes (A–C) and ECs (D–F) monocultured (A, B, D, E) or cocultured (C, F) showing the effect of exposure to a high glucose concentration and coculture and treatment with ARI. Pericytes or ECs were monocultured in the presence (B, E) or absence (A, D) of mitomycin C. For the coculture study, pericytes whose growth was arrested by mitomycin C were plated into 24-well plates, and on the after day ECs were seeded into the serum-free medium containing 5 or 30 mM d-glucose in the presence or absence of 10 µM ARI. Error bars indicate SE of five individual counts.
Inhibition of Pericyte Loss and EC Growth by ARI

growth spurt of ECs, it is likely that EC growth was controlled by the pericytes. Indeed, the prevention of pericyte loss by ARI resulted in the inhibition of EC progression. However, because ARI did not affect the mitotic activity of ECs cultured alone with 30 mM D-glucose, we considered that polyol accumulation was not the primary cause of the EC growth-inhibitory effect. The pericytes were directly rescued from high glucose-induced apoptosis by treatment with ARI, their levels of TGF-β were maintained, and consequently EC growth was inhibited.

Cocultures in the presence of 5 mM D-glucose showed growth inhibition of ECs and high levels of bioactive TGF-β, whereas cocultures with a high glucose concentration of 30 mM showed enhancement of EC growth and decreased levels of TGF-β. This result suggests that pericyte-mediated growth inhibition of ECs is dependent on TGF-β. In support of this, adding neutralizing antibodies against TGF-β to the culture media abolished the inhibitory effect on EC growth. Given that we used a porcine cell system and that our experiments paralleled those of Orlidge et al., the inhibitory effect of active TGF-β appears to be a general phenomenon, not one restricted to the bovine system. An increase in the glucose concentration to 30 mM did not alter the TGF-β levels in monocultures of ECs or pericytes; therefore, it is considered that pericyte loss in the presence of high glucose concentration is involved in the decrease of TGF-β levels and results in the inhibition of EC growth.

Orlidge et al. revealed that acid activation of TGF-β in serum-free media of pericyte or EC monocultures had an inhibitory effect on EC growth, whereas acid activation of coculture-conditioned media did not increase its inhibitory activity. This indicates that either ECs or pericytes in monoculture form latent TGF-β and that media conditioned by coculture of both cell types contain completely activated TGF-β. This was directly confirmed by ELISA assays. We also showed that the decreased TGF-β level in cells cocultured with 30 mM D-glucose was markedly increased by acid activation, suggesting the presence of latent TGF-β. The activation of TGF-β during coculture requires direct contact between pericytes and ECs and is mediated by the activity of plasminogen activator (PA)-plasmin, which is localized on the cell surface. Plasmin activates TGF-β by cleaving the TGF-β precursor, thus releasing active TGF-β. Activation of TGF-β can also be observed when PA activity is enhanced by exogenous basic fibroblast growth factor (bFGF). The disturbance of this process by these

### FIGURE 5

**Determination of the amount of bioactive TGF-β.** Culture supernatant was collected and the levels of bioactive TGF-β were measured directly by imunnoassay (Emax ImmunoAssay system). Acid treatment was carried out to convert the latent form of TGF-β in the culture media to the active form. Culture conditions were pericytes only, ECs only, coculture, and coculture with acid treatment. Values are mean ± SE of five different experiments.

### FIGURE 6

**Effect of supply of bioactive TGF-β and immunoabsorption with anti-TGF-β on EC proliferation in the presence of 5 mM (A) or 30 mM (B) D-glucose.** ECs were cultured with pericytes separately and together in serum-free media with or without a 1:1000 dilution of anti-TGF-β Abs/TGF-β at 1 ng/mL. Values are mean ± SE of five different experiments.

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even levels of AR expression are required. This is because AR functions during hyperglycemia, not normoglycemia. Taken together, it is suggested that polyols accumulate easily in pericytes and that the higher expression of AR renders pericytes more vulnerable to high glucose. D-glucose (metabolically inactive) at the same osmolality induced some extent of pericyte loss, but the difference with control (5 mM D-glucose) was insignificant. Therefore, it seems that the osmotic stress caused by increased AR activity, rather than the hyperosmolality of the culture medium, was responsible for the induction of apoptotic cell death.

Using a coculture system with exposure to a high glucose concentration, we demonstrated that the proliferation of ECs was accelerated as the number of pericytes gradually decreased. Because the decrease of pericytes preceded the
mechanisms may be associated with the attenuation of TGF-β activation in hyperglycemic conditions.

Our study demonstrated that high glucose exposure leads to the decreased production of bioactive TGF-β in retinal capillary cells. However, it is recognized that the increase in the expression of TGF-β, which stimulates ECM accumulation, is involved in the pathogenesis of diabetic nephropathy: The enhanced expression of TGF-β was observed in diabetic rats and humans. The TGF-β system was triggered by high glucose exposure, evidenced by the upregulation of TGF-β receptor and the activation of the downstream Smad signaling pathway. Moreover, the treatment of diabetic animals with a neutralizing anti-TGF-β antibody prevents the development of mesangial matrix expansion and the progressive decline in renal function. Interestingly, ARI prevents high glucose–induced increases in TGF-β in cultured mesangial cells. These findings suggest the relations between hyperglycemia and the levels of TGF-β are tissue specific.

Given that pericytes have little potential for replication in the adult retina, the prevention of pericyte loss by treatment with ARI is important. Although it is unlikely that pericyte loss and EC growth are essential to induce microaneurysm formation, our data raise the possibility that ARI has a potential to prevent the initial stage of the development of diabetic retinopathy. Indeed, it is reported that ARI-treated diabetic patients showed a slower rate of microaneurysm progression than controls. However, it should be noted that our findings cannot explain the mechanism responsible for neovascularization and capillary closure. The involvement of angiogenic factors such as bFGF and VEGF has been discussed in various reviews.

It is reported that the level of active TGF-β in the ocular vitreous of patients with diabetic retinopathy is decreased in comparison with controls, whereas the amount of total TGF-β does not differ significantly. The loss of antiangiogenic factors such as TGF-β may promote disordered angiogenesis. Accordingly, it would be valuable to determine whether a supply of TGF-β is essential for inhibiting the growth of ECs mediated by pericyte loss. The present study has demonstrated that the addition of bioactive TGF-β and treatment with ARI are able to prevent the proliferation of ECs accompanied by pericyte loss.

References


