Effect of High Glucose on Fibronectin Expression and Cell Proliferation in Trabecular Meshwork Cells

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PURPOSE. Increased fibronectin accumulation in the trabecular meshwork of glaucomatous eyes may contribute to the resistance of aqueous outflow and the development of primary open-angle glaucoma (POAG). Because the glucose level is increased in the aqueous humor of patients with diabetes, this study was conducted to determine whether a high-glucose condition alters fibronectin expression and contributes to cell loss in trabecular meshwork.

METHODS. The fibronectin mRNA level was determined using RT-PCR in bovine trabecular meshwork cells grown in normal (5 mM) or high (30 mM) glucose medium for 7 days, and cell counts were measured during this period. Distribution and the relative amount of fibronectin protein were determined in these cells by immunofluorescence microscopy and Western blot analysis.

RESULTS. Fibronectin mRNA level in cells grown in high-glucose medium was significantly upregulated two- to threefold compared with cells grown in normal medium (P < 0.05). In cells grown in high-glucose medium, fibronectin immunofluorescence was more intense, and the relative amount of fibronectin protein was significantly increased (131% ± 15% of control, P < 0.05) compared with the amount in cells grown in normal medium. A moderate decrease in cell number was observed in cells grown in high-glucose medium (78% ± 7% of control, P < 0.05).

CONCLUSIONS. These findings indicate that a high glucose level in aqueous humor of patients with diabetes may increase fibronectin syntheses and accumulation in trabecular meshwork and accelerate the depletion of trabecular meshwork cells, a characteristic feature of the outflow system in POAG. The striking similarity between high glucose–induced alterations in trabecular meshwork cells and those of vascular endothelial cells may represent a common biochemical link in the pathogenesis of POAG and diabetic microangiopathy. (Invest Ophthalmol Vis Sci. 2002;43:170–175)

Individuals with diabetes mellitus are reported to experience higher frequencies of glaucoma and elevated intraocular pressure.1–10 However, a common link in the pathogenesis of diabetic retinopathy and primary open-angle glaucoma (POAG) has not been identified. POAG, a progressive optic neuropathy, is generally associated with blockage of aqueous outflow and elevated intraocular pressure. Some have ascribed this pressure elevation to the accumulation of extracellular matrix (ECM) components, such as fibronectin and glycosaminoglycans in the aqueous outflow pathway.11,12 The trabecular meshwork in the chamber angle is thought to function as a self-cleaning filter and to participate in the regulation of aqueous humor outflow and the control of intraocular pressure.13 This specialized tissue is composed of sheets of trabecular beams made up of various ECM elements.14–20 Lining the beams are trabecular meshwork cells involved in the maintenance of the normal outflow system. Connective tissue in the trabecular beams contains ECM proteins, including fibronectin, laminin, heparan sulfate, and collagens types I, III, IV, V, and VI.14–20

The increased expression of laminin21 and fibronectin22 in dexamethasone-treated trabecular meshwork cells mirrors the increased fibronectin and laminin deposition in the aqueous outflow pathway that has been shown to occur in elderly and glaucomatous eyes.12,15 Increased accumulation of ECM proteins, including fibronectin, a major component of the aqueous humor, may particularly influence the flow of aqueous humor through the juxtacanalicular trabecular meshwork and the inner walls of Schlemm’s canal. It is believed that changes in either the cell activities or the makeup of the trabecular meshwork matrix may adversely affect aqueous outflow, leading to intraocular pressure elevations and glaucomatous conditions.

Davies et al.23 have reported that the glucose levels in aqueous humor of patients with diabetes were significantly higher (3.2 mM vs. 7.8 mM) compared with levels in persons without diabetes. We observed a two- to threefold increase in aqueous glucose levels in diabetic rats compared with those of control rats. Because trabecular meshwork cells are in constant contact with aqueous humor, it is likely that changes in the aqueous humor’s constituents may influence biochemical functions of these cells. Several groups, including ours, have found that the hyperglycemic milieu induces fibronectin overexpression in the vascular endothelium. In human endothelial cells grown in high-glucose medium, tissues from diabetic animals, and retinal capillaries of patients with diabetes, we have found fibronectin overexpression24–26 known to be associated with the development of vascular basement membrane thickening, a prominent abnormality in diabetic microangiopathy.

A number of investigators have reported that the intraocular pressure in persons with diabetes tends to be higher than in those without diabetes and that the incidence of glaucoma is two to three times greater in persons with diabetes than in persons of similar age without diabetes.1–10 Although the reason for increased incidence of open-angle glaucoma in persons with diabetes has not been elucidated, it is likely that diabetes-associated changes in the trabecular ECM may contribute to decreased aqueous outflow. Furthermore, studies have demonstrated a decrease in trabecular meshwork cellularity in patients with POAG that is similar to the endothelial cell loss in diabetic retinopathy.27 Recently, the chemoattractant potential of fibronectin in aqueous humor was reported to play a role in trabecular meshwork cell loss in glaucomatous eyes.28 Other investigators have reported depletion of trabecular meshwork cells in patients with POAG.29,30 To the best of our knowledge, there has been no report on diabetes-induced ECM changes or

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Supported by a Research Award from the American Diabetes Association, the National Eye Institute, and in part by departmental grants from Research to Prevent Blindness, Inc., and the Massachusetts Lions Eye Research Fund Inc.

Submitted for publication April 19, 2001; revised August 9, 2001; accepted September 5, 2001.

Commercial relationships: N.

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accumulation in the trabecular meshwork. In this study, we have examined whether a high-glucose condition modulates fibronectin expression and trabecular meshwork cell proliferation that could lead to increased fibronectin accumulation and cell loss in the trabecular meshwork.

**Materials and Methods**

**Cell Culture**

Trabecular meshwork cells were grown from bovine trabecular tissues as described\(^1,2,3\) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS; Sigma, St. Louis, MO) and antibiotics (100 U/mL penicillin and streptomycin). Briefly, the anterior segments were removed and under a dissecting microscope, thin strips of trabecular meshwork were carefully isolated and placed on 35-mm Petri dishes. These explants were fed with complete DMEM containing 10% FCS. Cells from the explants appeared within 2 to 3 days with ruffled edges, numerous cell extensions, and overlapping processes typical of trabecular meshwork cells. To determine the effect of high glucose, trabecular meshwork cells from passage 2 to 4 were grown to semiconfluence and exposed to normal (5 mM) or high (30 mM) D-glucose medium for 7 days. In each experiment, at least four different trabecular meshwork cell isolates were examined.

**Cell Count**

In each cell count experiment, an equal number of cells (50,000/35-mm well) were seeded at the start of the experiment for both conditions: cells grown in normal medium or high-glucose medium. Cell counts were monitored at each time point by performing duplicate counts. Two time points were assessed after exposure to high glucose on days 4 and 7. At semiconfluence, cells grown in normal or high-glucose medium were washed twice with Ca-free PBS, trypsinized, and counted in duplicate in a cell counter (Coulter Electronics, Inc., Hialeah, FL) for each experiment.

**Reverse Transcription–Polymerase Chain Reaction**

In each experiment RNA from cells grown in normal medium was processed in parallel with RNA from cells grown in high-glucose medium. Reverse transcription was performed in a 20-μL volume, with 1 μg RNA, 200 U reverse transcriptase, 2.5 μM random hexamers, 1 mM each dNTP, 5 mM MgCl\(_2\), PCR buffer, and RNase inhibitor, for 10 minutes at room temperature, followed by 40 minutes at 42°C. At the end of reverse transcription, samples were heated to 95°C for 5 minutes, cooled on ice, and treated with RNase H (1 U) for 15 minutes at 37°C. The protocol for PCR was designed to measure the level of fibronectin expression in relation to the expression of an endogenous internal standard gene, β-actin. To prevent quantitative inaccuracies deriving from competitive effects and different efficiency and ranges of amplification of the two cDNAs, the fibronectin and β-actin cDNAs generated in the same reverse transcription reaction were amplified in separate tubes containing increasing volumes of the reverse transcription reaction (1, 2, and 4 μL) to document amplification in the linear region for each cDNA. The primers used to amplify the fibronectin and β-actin (Table 1) were designed from published sequences.\(^5,6\) The specificity of the PCR was enhanced by using the hot-start approach.\(^7\) The PCR, containing the appropriate aliquot of reverse transcription material with 0.2 μM of each primer, 2.5 U DNA polymerase (AmpliTaq; Roche Molecular Biochemicals, Indianapolis, IN), MgCl\(_2\) (1.5 mM for fibronectin, 1.8 mM for actin), and PCR buffer in a 50-μL volume, was performed in a DNA thermal cycler (Hybaid, Middlesex, UK) using the following cycle conditions: denaturation for 1 minute at 95°C for both fibronectin and actin, annealing for 1 minute at 54°C for fibronectin, and 1 minute at 57°C for actin, and extension for 2 minutes at 72°C for both fibronectin and actin. PCR was performed with 26 cycles for fibronectin and 22 cycles for β-actin.

**Analysis and Quantitation of PCR Products**

PCR products from cells grown in normal or high-glucose medium were always resolved on the same gel (1.0% agarose) containing 0.05 μL/mL GelStar, a DNA-binding dye (BMA, Rockland, ME) together with molecular weight markers (100-bp DNA ladder; Gibco, Grand Island, NY). Positive identification of the RT-PCR products for fibronectin was confirmed by Southern blot hybridization using a rat fibronectin cDNA. After electrophoresis, the gel was photographed (Positive/Negative Instant Film 665; Polaroid, Cambridge, MA) and signal intensity was quantitated at nonsaturating exposure of the film with a soft laser scanning densitometer (Zeineh; Biomed Instruments, Chicago, IL), as previously described.\(^8\) The densitometric values of the PCR products generated from increasing volumes (1, 2, and 4 μL) of reverse transcription reaction represented linear amplification. These values were averaged to yield the fibronectin and actin signals for each sample and expressed as densitometric units per microliter of reverse transcription reaction. The linearity of amplification verified that experimental data represented PCR products from the exponential phase of the reaction.

**Immunofluorescence Microscopy**

To study the distribution pattern and relative amounts of fibronectin, immunofluorescence staining for fibronectin was performed with bovine trabecular meshwork cells grown in normal or high-glucose medium. Briefly, cells grown to confluence were fixed in 1% paraformaldehyde for 45 minutes, washed in PBS, and treated with 2% BSA for 15 minutes to block nonspecific antibody binding. The cells were then incubated overnight at 4°C in a moist chamber with a polyclonal rabbit antirat fibronectin antibody (Chemicon, Temecula, CA) diluted 1:100 in PBS containing 2% BSA. After three PBS washes the cells were incubated for 1 hour with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma) diluted 1:100 in PBS containing 2% BSA. After three PBS washes, coverslips were mounted in antifade medium (Slow-Fade; Molecular Probes, Eugene, OR). Negative control samples were processed in exactly the same way as those in the experimental groups except that the primary antibody was omitted. The cells were viewed and photographed using a confocal microscope (LSM510; Carl Zeiss, Jena, Germany) equipped with software (LSM510-v2.01; Carl Zeiss). Investigators without knowledge of treated or control samples scored for fluorescence intensity in cells grown in normal

**Table 1. Primers Used for DNA Amplification of Fibronectin and β-Actin**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Size of Amplified Fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine fibronectin-U</td>
<td>5’-TATGACGATGGGAA-3’</td>
<td>257</td>
</tr>
<tr>
<td>Bovine fibronectin-D</td>
<td>5’-CTGTCAGCCTGTTACA-3’</td>
<td>257</td>
</tr>
<tr>
<td>Bovine β-actin-U</td>
<td>5’-GAACTGGGAGCGCAT-3’</td>
<td>433</td>
</tr>
<tr>
<td>Bovine β-actin-D</td>
<td>5’-CAAGGCGACGTGACA-3’</td>
<td>433</td>
</tr>
</tbody>
</table>

Specific 5’ and 3’ primers used for DNA amplification of fibronectin and β-actin by polymerase chain reaction are shown. The expected sizes of the amplified bands are also indicated. U and D represent upstream and downstream primers, respectively.
or high-glucose medium. On a scale of 0 to 4, background fluorescence from negative control cells without antibody was scored as 0 and the highest fluorescence intensity as 4.

**Gel Electrophoresis and Western Blot**

Cells exposed to normal or high-glucose medium were washed with PBS and lysed with buffer containing 10 mM Tris (pH 7.5; Sigma), 1 mM EDTA, and 0.1% Triton X-100 (Sigma). Cellular protein content in the cell extract was measured by Bradford’s method. After addition of an equal volume of 2X sample buffer and denaturation at 95°C for 5 minutes, the extracts (containing 10 μg of protein) were electrophoresed at 200 V for 45 minutes. Molecular weight (Bio-Rad, Richmond, CA) and bovine placenta fibronectin (Sigma) standards were included in separate lanes in each gel. After electrophoresis, the proteins were transferred onto nitrocellulose membrane (Bio-Rad) according to the procedure of Towbin et al., using a semidry apparatus with the Towbin buffer system. Western blot analysis was performed to examine the steady state level of fibronectin expression in these cells. The proteins transferred to the nitrocellulose membrane were detected with a chemiluminescence system (Immun-Star Chemiluminescent Protein Detection System; Bio-Rad) and the rabbit anti-human fibronectin antiserum (Chemicon). Briefly, the membrane was blocked with 5% nonfat dry milk for 1 hour, and incubated with rabbit anti-rat fibronectin antibody solution (1:500) in 0.2% nonfat milk for 1 hour. The blot was washed with Tris-buffered saline containing 0.1% Tween 20 and then incubated with antibody solution containing goat anti-rabbit IgG antibody conjugated with alkaline phosphatase enzyme (Sigma) for 1 hour. The membrane was washed as above, applied to the Immun-Star chemiluminescent substrate, and exposed to x-ray film (Fuji; Tokyo, Japan). Densitometric analysis of the luminescent signal was performed at nonsaturating exposures with a laser scanning densitometer.

**Statistical Analysis**

All data are expressed as mean ± SD. Differences in mean cell number, and densitometric values were compared using one way analysis of variance (ANOVA) combined with Student-Newman-Keuls test for multiple comparisons. *P < 0.05* was considered statistically significant.

**RESULTS**

Bovine trabecular meshwork cells grown in high-glucose medium for 7 days exhibited increased fibronectin expression at both the mRNA and protein levels. The fibronectin mRNA level determined by RT-PCR in cells grown in high-glucose medium was significantly increased two- to threefold (250% ± 54% of control, *P < 0.05, n = 4*) compared with cells grown in normal medium (Figs. 1A, 1B), whereas the actin mRNA level, used as an internal control, remained unchanged in cells grown in normal or high-glucose medium (98% of control). The effect of high glucose on fibronectin mRNA expression was similar in cells from passages 2 and 4. The densitometric values of the RT-PCR amplified products increased linearly, with an increasing amount of reverse transcribed cDNA (1, 2, and 4 μL) for both fibronectin and actin, which indicates that PCR products were derived from the exponential phase of the experiment. Western blot analysis in corresponding cell cultures indicated that fibronectin protein level in cells grown in high-glucose medium was also significantly increased (131% ± 15% of control, *n = 4, P < 0.05*) compared with cells grown in normal medium (Figs. 2A, 2B). Taken together, these data indicate that a high glucose level upregulated fibronectin expression in trabecular meshwork cells.

Distribution and relative quantity of fibronectin in trabecular meshwork cells was determined by immunostaining and fluorescence microscopy. In cells grown in high-glucose medium the fluorescence pattern from FITC-labeled fibronectin antibody suggested the presence of fibronectin as an organized scaffold on and around the cells. In general, we observed that the cell cytoplasm stained positively for fibronectin, and the immunofluorescence was more intense in the cells grown in high-glucose medium compared with cells grown in normal medium. Semiquantitative analysis of fibronectin protein level based on fluorescence intensity yielded a score of 1.6 ± 0.7 for cells grown in normal medium and 3.4 ± 0.5 for cells grown in high glucose medium, indicated a significant increase (*P < 0.05*) in fibronectin expression in cells grown in high-glucose medium; omission of the primary antibody showed almost no signal (Figs. 3A-C).

The effect of high glucose on cell proliferation was examined in cultures of trabecular meshwork cells grown in normal or high-glucose medium for up to 7 days. A growth assay conducted to determine the cell proliferation profile of the trabecular meshwork cells indicated that these cells take approximately 5 days for cell doubling. The result confirmed our previous observation and report by other investigators. **Figure 1.** (A) RT-PCR analysis of fibronectin mRNA level in bovine trabecular meshwork cells exposed to normal or high-glucose medium. PCR amplification products for fibronectin and β-actin as detected on GelStar-stained agarose gel with 1-, 2-, and 4-μL samples of reverse-transcribed cDNA. PCR with a fibronectin primer pair generated a single 257-bp band and with the β-actin primer pair generated a single 433-bp band. (B) Effect of high glucose on the fibronectin mRNA level in bovine trabecular meshwork cells. Cells were grown in normal or high-glucose medium for 7 days, and fibronectin mRNA level determined using RT-PCR. Data are presented as mean ± SD and are expressed as a percentage of control after normalizing to β-actin mRNA level (*n = 4; *P < 0.05*).
DISCUSSION

In this study we determined that the cellular changes induced by high glucose in trabecular meshwork cells were strikingly similar to those reported in vascular endothelial cells. In line with our current finding that high glucose induces fibronectin overexpression at both the mRNA and protein levels in trabecular meshwork cells, previous studies reported similar observations in vascular endothelial cells grown in high-glucose medium. The high glucose–induced inhibition of trabecular meshwork cell proliferation was also similar to that observed in vascular endothelial cells grown in a high-glucose condition. The changes in fibronectin expression observed in this study using 30 mM glucose concentration is likely to occur with a more physiological high glucose level, such as 15 mM glucose, but may take a longer time. Studies on cell proliferation showed that a 20-mM glucose condition significantly inhibits microvascular endothelial cell proliferation after 7, 10, and 14 days of exposure; however, the antiproliferative effect is more pronounced with increasing duration of exposure to a high-glucose condition.

Although it is known that the trabecular meshwork represents the principal site of aqueous outflow from the anterior chamber, the mechanism regulating aqueous outflow is unclear. When aqueous fluid cannot exit the eye as rapidly as it is produced, the pressure in the eye increases, and the high intraocular pressure is believed to damage the optic nerve. In open-angle glaucoma, the angle formed between the iris and the cornea remains open, and the elevated pressure is caused at least in part by blockage of aqueous outflow due to changes in the trabecular meshwork. Studies indicate that fibronectin is present in increased amounts in the human trabecular meshwork drainage channels of glaucomatous eyes. Increased fibronectin content in the drainage outflow system of the trabecular meshwork may impair the regulation of outflow.

FIGURE 2. (A) Western blot analysis of fibronectin protein level in bovine trabecular meshwork cells. Cells were grown in normal medium (lanes 1, 2, and 3) or in high-glucose medium (lanes 4, 5, and 6) for 7 days, and cell extracts were used for Western blot analysis. A 230-kDa fibronectin band was observed. Molecular weight of the fibronectin band was confirmed from molecular weight markers that were run in parallel. (B) Effect of high glucose on fibronectin protein level in bovine trabecular meshwork cells. Fibronectin protein level was determined using Western blot analysis in cells grown in normal or high-glucose medium. Data are presented as mean ± SD and expressed as a percentage of control (n = 4; *P < 0.05).

FIGURE 3. Fibronectin immunoreactivity in bovine trabecular meshwork cells. Representative photomicrographs of (A) negative control cultures without primary antibody, (B) cells grown in normal medium, and (C) cells grown in high-glucose medium. The fibronectin immunofluorescence was more intense in cells grown in high-glucose medium than in cells grown in normal medium. Magnification, × 210.
mechanism and contribute to the abnormally high resistance in POAG. The connective tissue composition of the trabecular meshwork is similar to other highly compliant and resilient tissues, such as the conjunctiva and is a determinant of its mechanical properties. Previous histologic examinations have shown that during development of glaucoma, fibronectin accumulation occurs in the trabecular meshwork and in the endothelial lining of Schlemm’s canal.12-15

It is widely believed that the trabecular meshwork, which functions as a valve structure, must be able to alter its configuration with changes in intraocular pressure and be sufficiently resilient to return to baseline condition at resting stage.43 Such resiliency of the trabecular tissue may be compromised by changes in the composition of the structural components of the ECM.45 The presence of fibronectin in the trabecular meshwork is implicated in cell-ECM interactions at this site, and its abnormal accumulation in glaucomatous eyes may signify a structural compromise in the function of the trabecular cells in the outflow system.45 Because glucose levels are significantly higher (2.5-fold) in aqueous humor of persons with diabetes compared with those in normal subjects,29 and the trabecular meshwork cells are exposed to the high-glucose milieu from the aqueous humor, it is likely that fibronectin expression is upregulated in the trabecular meshwork of diabetic eyes. It is thus possible that overexpression of fibronectin in the trabecular meshwork cells of patients with diabetes may play a role in the resistance of aqueous outflow and contribute to the development of POAG.

The source of aqueous fibronectin is still unknown, and the mechanism by which fibronectin concentration increases in glaucoma is not well understood. However, our current findings suggest that an increase in local production of fibronectin by the trabecular meshwork cells may occur in diabetes and contribute to blockage in the aqueous outflow pathway. Previously, we have shown that high glucose and diabetes upregulate fibronectin expression in microvascular endothelial cells in culture, human endothelial cells, and retinal capillaries of patients with diabetes.24,46,47 Such increased fibronectin synthesis and deposition are associated with structural and functional changes relevant to the development of lesions of diabetic retinopathy.24,46 These findings may begin to explain the association between diabetes and elevated intraocular pressure that has long been considered.5-8

The depletion of trabecular meshwork cells is a characteristic feature of the outflow system in POAG. The meshworks of patients with POAG have been reported to have a lower number of cells per unit tissue area than age-matched normal control subjects.29,50 It has been suggested that the loss of trabecular meshwork cells in POAG could result in a reduced outflow facility.25 It is of interest that in diabetic retinopathy, both pericytes and endothelial cells of the retinal capillaries are also lost, with subsequent development of acellular capillaries, a prominent and fundamental lesion in the retina.50,51 In this study we have determined that trabecular meshwork cells grown in a high-glucose condition exhibit reduced cell proliferation similar to pericyte and endothelial cell proliferation under a high-glucose condition.50,51 The reduced proliferation of the trabecular meshwork cells induced by the high glucose level may be linked to the excess fibronectin synthesis. Therefore, it is possible that the decrease in cell number observed in trabecular meshwork during development of glaucoma may be exacerbated, at least in part, by the high glucose concentration present in aqueous humor of patients with diabetes.

This study demonstrates for the first time that a high glucose level induces fibronectin overexpression in trabecular meshwork cells and may contribute to excess fibronectin accumulation in the trabecular meshwork. High glucose-induced fibronectin upregulation may be a common biochemical link that on the one hand contributes to the development of thickened vascular basement membranes in diabetic microangiopathy and on the other hand alters the structural content, compromises resiliency, reduces cellularity, blocks aqueous outflow in trabecular meshwork and leads to the development of POAG in persons with diabetes. Our findings could serve as a basis for understanding the role of high glucose-induced abnormal ECM accumulation in glaucoma and diabetes.

References

High Glucose Effect in Trabecular Meshwork Cells


