High Glucose–Induced Downregulation of Connexin 43 Expression Promotes Apoptosis in Microvascular Endothelial Cells

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PURPOSE. This study aims to determine whether high glucose–induced inhibition of connexin 43 (Cx43) expression and reduced gap junction intercellular communication (GJIC) promote microvascular endothelial cell loss.

METHODS. To downregulate Cx43 protein expression in rat microvascular endothelial cells (RMECs), the cells were grown in high (30 mM) glucose medium for 7 days, or transfected with antisense-Cx43 (AS-Cx43) oligonucleotides. Western blot analysis confirmed significant inhibition of Cx43 protein expression. Scrape load dye transfer (SLDT) assay showed significant reduction in GJIC activity in these cells compared to cells grown in normal medium or transfected with random oligonucleotides. In parallel, Cx43 immunostaining showed significant decrease in number of Cx43 plaques in cells with reduced Cx43 expression. DNA ladder assay, TUNEL assay, and differential staining were performed to identify cells undergoing apoptosis.

RESULTS. DNA ladder analysis, TUNEL assay, and differential staining indicated a significant increase in the number of apoptotic cells when Cx43 protein expression was reduced in both high-glucose cells or cells transfected with AS-Cx43 oligonucleotides with concomitant downregulation of GJIC activity. Additionally, DNA fragmentation, which was evident in cells with reduced Cx43 expression, suggested early phases of apoptosis.

CONCLUSIONS. These results provide the first evidence that high glucose–induced downregulation of Cx43 expression is an early trigger for inducing apoptosis in microvascular endothelial cells. This finding may have important implications toward breakdown of vascular homeostasis and initiation of apoptosis in diabetic retinopathy. (Invest Ophthalmol Vis Sci. 2009;50: 1400–1407) DOI:10.1167/iovs.07-1519

Intercellular communication through gap junctions plays a critical role in the maintenance of tissue homeostasis.¹ Gap junction channels are composed of two hemi-channels that dock together and form an active channel between adjacent cells. These channels are composed of connexin subunits and allow transfer of ions and small molecules.²,³ In most tissues, including the retina, adjacent cells communicate through gap junctions involving connexins. Connexin 43 (Cx43) is abundantly present in the retina, which suggests a substantial amount of Cx43-mediated gap junctional coupling. Changes in Cx43 expression and gap junction activity have been associated with a wide variety of pathologic conditions and diseases,⁴–⁶ including marked reduction of gap junction activity in the diabetic retinal vasculature.⁷ Previously, we reported that Cx43 expression is significantly down-regulated by high glucose in rat microvascular endothelial cells (RMECs) and retinal pericytes.⁸,⁹ The implication of down-regulation of Cx43 expression and reduced gap junction intercellular communication (GJIC) may be important in the context of diabetic retinopathy.

The consequence of compromised GJIC activity in RMECs is not well understood. To maintain vascular homeostasis, cells communicate with neighboring cells via gap junctions and exchange various small molecules, including second messengers such as nucleotides, cAMP, IP₃, and Ca²⁺.¹⁰,¹¹ Such exchange of molecules is thought to coordinate appropriate response to external stimuli.¹² Modulation in various connexin gene expressions has been shown to regulate gap junctional permeability and trafficking of signaling molecules between adjacent cells. To maintain homeostasis, a delicate balance between the number of cells dying and the number of cells proliferating is essential. To that effect, several studies have shown that changes in GJIC activity can influence cell survival or apoptosis.¹²–¹⁵ In rat ventricular myocytes, reduced Cx43 expression resulted in increased apoptosis.¹³ Similarly, in mammary epithelial cells, reduced connexin 26 expression resulted in increased apoptosis,¹⁶ and massive apoptosis was observed in vascular cells of Cx45-deficient mice.¹⁷ Cx43-null mice exhibited increased programed cell death,¹⁸ and blockage of testicular connexins in rat seminiferous epithelial cells induced apoptosis.¹⁹ Overall, these studies indicate that alteration in connexin expression profoundly affects cell survival.

In diabetic retinopathy, retinal microvascular cell loss is a characteristic and early lesion that occurs by apoptosis.²⁰,²¹ Despite some progress in understanding how vascular cells may be lost in diabetic retinopathy, the biochemical and molecular mechanisms underlying the accelerated cell loss in retinal capillaries in diabetic retinopathy are not well understood. In particular, the upstream events that trigger apoptosis in diabetes are unclear. While some studies indicate that the expression of Cx43 and GJIC activity in retinal microvascular cells is inhibited by high glucose or hyperglycemic condition,²⁰,²¹ the consequence of reduced Cx43 expression and GJIC has not been determined in retinal microvascular cells. We hypothesize that high glucose–induced downregulation of Cx43 expression reduces GJIC activity and contributes to RMEC loss by apoptosis. In this study, we have determined the consequence of high glucose-induced inhibition of Cx43 expression on GJIC activity and cell survival in RMECs, and corroborated the effect of reduced GJIC in retinal endothelial cells.
**Materials and Methods**

**Cell Culture**
RMECs from epididymal fat pads ascertained positive for von Willebrand factor (vWF) by immunofluorescence microscopy were used in this study. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS, from Sigma, St. Louis, MO) with antibiotics and antimycotics in normal (5 mM) medium or high (30 mM) glucose medium for 7 days. To determine the effect of reduced Cx43 expression and GJIC activity, at subconfluency cells were transfected with AS-Cx43 designed to reduce Cx43 expression. Similarly, the effect of reduced gap junction activity was studied in rat retinal endothelial cells (RRECs). Retinas (n = 12) freshly isolated from rats were cut into small pieces (approximately 2 mm × 2 mm) in PBS under sterile condition, and subjected to 0.2% collagenase type II (Worthington, Lakewood, NJ) digestion with gentle shaking for 1.5 hours at 37°C. During incubation, care was taken to keep the minced tissue from forming into a pulp. To keep the minced tissue and the capillary fragments loose, the mixture was passed few times through a 200 µm pipette tip. After collagenase digestion, the solution containing the loose capillaries was washed over a sterile 110 µm nylon mesh, and the filtrate containing small capillaries that passed through the nylon mesh was then centrifuged at 1000g. The pellet was then resuspended in complete media containing DMEM, antibiotics, antimycotics, and 10% FBS. This mixture was plated in two 35 mm dishes and allowed to grow for 2 weeks, with medium change every alternate day. Generally, by 2 weeks, the RRECs reach 60% to 70% confluency. Endothelial cell characteristics were confirmed based on “cobblestone” morphology and vWF labeling. RRECs used in this study were from passage two to five.

**Cell Transfection with Antisense Cx43 Oligonucleotide**
RMECs plated in normal medium and grown to subconfluency were transfected with AS-Cx43 or random oligonucleotide (Oligos, Etc., Wilsonville, OR). Cells were transfected in reduced serum medium (OptiMem; Invitrogen, Carlsbad, CA) in the presence of 8 µM reagent (Lipofectin; Gibco, Grand Island, NY) and 0.1 µM of either AS-Cx43 or random oligonucleotides for 4 hours and then returned to normal medium. The cells were analyzed 72 hours after transfection. The sequence for AS-Cx43 oligonucleotide was 5'-AGTCCACCCATGTCGTCCT-3' and random oligonucleotide was 5'-TATGGTACGTGTCGTCCT-3'; the AS-Cx43 oligonucleotide sequence was designed from a published sequence for Cx43 (accession number x06656.1). The antisense oligonucleotide was targeted against the translation initiation site of the Cx43 transcript. The effiency of the antisense-Cx43 oligonucleotides were tested at different concentrations between 0.01 µM to 0.5 µM. Approximately 50% reduction in Cx43 expression was achieved at the 0.1 µM concentration, which was used in this study since high-glucose exposure reduces approximately 50% Cx43 expression. Both the oligonucleotides were phosphorothioate modified to impart stability.

**Gel Electrophoresis and Western Blot Analysis**
To isolate protein, RMECs were washed in PBS and lysed with buffer containing 10 mM Tris (pH 7.4; Sigma), 1 mM EDTA, and 0.1% Triton X-100 (Sigma). Protein content in cell lysates was determined by bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL). To determine the steady state level of Cx43 protein, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 15 µg of protein per lane. The samples were then transferred onto nitrocellulose membrane according to Towbin’s procedure. Briefly, the membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 2 hours and incubated with mouse anti- rat Cx43 antibody (Chemicon International, Temecula, CA) solution (1:500) in 0.2% nonfat dry milk overnight at 4°C. After three washes in TBS containing 0.1% Tween-20, the membranes were incubated with goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma) for 1 hour. The membranes were then washed as described above, applied to the chemiluminescent substrate (Immun-Star; BioRad Laboratories, Hercules, CA) and exposed to x-ray film (Fujiﬁlm, Tokyo, Japan). Densitometric analysis of Western blot signals was performed at non-saturating exposures using imaging software (NIH Image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). To identify membrane-bound Cx43 from control and high-glucose samples, membrane fractions were prepared in ice-cold alkaline hypotonic cell lysis buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM iodoacetamide, and 1 mM phenylmethylsulfonyl fluoride, briefly sonicated, and centrifuged at 13,000g. The resulting pellets were dissolved in 2% sodium dodecyl sulfate (SDS) and the protein subjected to Western blot analysis as described elsewhere.25 (NIH Image or ImageJ software).

**TUNEL and Immunostaining**
TUNEL assay was performed using the (ApopTag in situ Apoptosis Detection kit; Chemicon) according to the manufacturer’s instruction. Briefly, cells were ﬁxed in 1% paraformaldehyde in PBS (pH 7.4), and permeated with pre-cooled mixture of ethanol and acetic acid mixed at 2:1 ratio. After three washes with PBS, cells were incubated with equilibrium buffer and then incubated with TdT enzyme in a moist chamber. Cells were then washed with PBS and incubated with anti-digoxigenin-labeled dUTP using terminal transferase and mounted with DAPI. Digital images were recorded for analysis, and then the cells were counterstained with Cx43 antibody. Briefly, cells were blocked with 2% bovine serum albumin in PBS for 15 minutes and then incubated with mouse anti-rat Cx43 antibody (Chemicon) solution (1:100) overnight at 4°C in a moist chamber. After three washes of PBS, the cells were incubated with goat anti-mouse IgG conjugated with a ﬂuorescent stain (Rhodamine-red; Jackson Immunoresearch Laboratories, West Grove, PA) for 1 hour. After three PBS washes, the coverslips were mounted in an anti-fade reagent (SlowFade; Molecular Probes, Eugene, OR). Negative control samples were processed by the same procedure except that the primary antibody was omitted. The cells were viewed and images digitally recorded using a confocal microscope (LSM 510; Zeiss, Göttingen, Germany). Cx43 signals appeared as fluorescent punctate dots and were present between adjacent RMECs. At least 10 random ﬁelds were scored for the punctate dots.

**Scrape Load Dye Transfer Technique (SLDTR)**
SLDTR was used to assess GJIC activity. Briefly, the confluent RMECs on coverslips were washed three times with PBS containing 0.01% Ca2+ and Mg2+. An aliquot of 1.5 mL of PBS containing 0.05% fluorescent dye ( Lucifer yellow; Molecular Probes) was added to cover the coverslips and random cuts were made on cell monolayers of each coverslip. The cells were incubated for 3 minutes at room temperature and then rinsed three times with PBS containing 0.01% Ca2+ and Mg2+. The cells were ﬁxed with 4% paraformaldehyde in PBS and photographed with a digital camera attached to a ﬂuorescence microscope (Diaphot; Nikon, Tokyo, Japan). The number of dye-coupled cell layers on either side of the scrape was counted in at least ten random areas to evaluate GJIC activity.

**Differential Staining Assay for Assessment of Apoptotic Cells**
Differential staining assay was performed using acidine orange and ethidium bromide dye mixture to identify apoptotic cells. Briefly, RMECs grown on coverslips were washed with PBS, exposed to a dye mixture containing 25 µg/mL acidine orange and 25 µg/mL ethidium bromide for 3 minutes, washed in PBS, ﬁxed in 4% paraformaldehyde, and then mounted. Under fluorescence microscopy, apoptotic cells appeared bright green and exhibited occasionally fragmented chromatin, whereas necrotic cells (non-viable) stained uniformly and appeared orange. At least ten random ﬁelds were selected from each group and 200 cells counted per ﬁeld. Number of cells undergoing apoptosis was expressed as a percentage (number of apoptotic cells divided by total number of viable cells per ﬁeld).
DNA Ladder Assay
DNA isolation and subsequent DNA ladder assay were performed according to the method described by Gong et al. Briefly, for each group of cells, approximately 2 million cells were harvested, centrifuged, and pellets resuspended in 40 μL of phosphate-citric buffer containing 192 parts of 0.2 M NaH2PO4, and 8 parts of 0.1 M citric acid (pH 7.8) at room temperature for 30 minutes. After incubation, cells were centrifuged and the supernatant was transferred into new tubes. The supernatant was then exposed to 3 μL of 0.25% nonionic non-denaturing detergent (Nonidet NP-40; Sigma) and 3 μL of 1 mg/mL RNase A (Sigma) for 30 minutes at 37°C. This was followed by incubation in the presence of 1 mg/mL proteinase K for 30 minutes at 37°C. The extracted DNA was subjected to electrophoresis in 1.8% agarose gel with 1.25 μg/mL gel, a sensitive dye (GelStar; BMA, Rockland, ME) for identification of DNA fragments. The gel was photographed with positive instant film (667; Polaroid, Cambridge, MA) and analyzed with NIH Image software (NIH).

Treatment of Cells with Beta Glycyrrhetinic Acid (GA), a GJIC Inhibitor
Cells were grown for 7 days in normal medium, high-glucose medium, or high-glucose medium exposed to βGA or βglycyrrhizic acid (GZ; inactive analog of the inhibitor). The cells were exposed to βGA at a concentration of 50 μM or 100 μM for 16 hours before harvesting. Parallel control experiments were performed using the exact set up except that a group of cells were exposed to 50 μM GZ, the inactive analog of the inhibitor β-GA.

Statistics
All data were expressed as mean ± SD. Comparisons between groups were performed with one-way ANOVA and Student’s t-test. A value of P < 0.05 was considered statistically significant.

RESULTS
Effect of High Glucose-Induced Cx43 Downregulation on Microvascular Endothelial Cell Apoptosis
To determine whether high glucose-induced downregulation of Cx43 expression contributes to accelerated cell death, Western blot analysis was performed to determine Cx43 protein level; DNA laddering, TUNEL, and differential staining assays were performed to identify apoptotic cells. In line with our previous findings, RMECs grown in high-glucose medium showed significant reduction in Cx43 protein expression compared to cells grown in normal medium (59.4% ± 26% of control, P = 0.001, n = 9; Fig. 1A). Western blot analysis performed with protein from the cytosolic or the membrane-bound fraction showed a significant reduction in Cx43 protein level in HG cells compared to that of normal cells for both fractions (40% ± 8% of normal, P < 0.001 in the cytosolic fraction; 43.5% ± 7% of normal, P < 0.002 in the membrane-bound fraction). Analysis of genomic DNA fragmentation assay revealed increased band intensity in the DNA ladder, indicating increased apoptosis in cells grown in high-glucose medium compared to cells grown in normal medium (151.6% ± 19% of control, P = 0.005, n = 5; Figs. 1B, 1C). Differential staining indicated a significantly higher percentage of cells with condensed chromatin or fragmented DNA (206% ± 27% of control, P < 0.001, n = 5; Fig. 1D), and TUNEL assay indicated a significantly higher number of TUNEL-positive cells when cells were grown in high-glucose medium (7.3 ± 1.6 vs. 3.7 ± 1.4, P = 0.004; n = 4) (Fig. 1E). The intensity change in DNA ‘ladder’ was evident much before morphologic evidence of cell death was seen by microscopy. In addition, we observed that cells exhibiting very low levels of Cx43, as determined from Cx43 immunostaining, were not only apoptotic but appeared to be surrounded by cells with diminished levels of Cx43 immunoreactivity indicative of reduced GJIC activity (Fig. 2C). Taken together, the results suggest high glucose-induced inhibition of Cx43 expression is closely associated with increased apoptosis in RMECs.

Effect of Cx43 Downregulation on Gap Junction Activity in RMECs
To determine the effect of reduced Cx43 expression on cell-cell communication, we used AS-Cx43 oligonucleotides to selectively reduce Cx43 expression, and then examined its effect on GJIC activity using SLDT assay. Western blot analysis indicated a significant decrease in Cx43 protein level in RMECs transfected with AS-Cx43 oligonucleotides compared to the untransfected control cells or cells transfected with random oligonucleotides (76% ± 12% of control, P = 0.004; 65% ± 14% of random, P = 0.02, respectively, n = 6). No significant difference in Cx43 protein level was observed between untransfected control cells and random oligonucleotide transfected cells (118% ± 28% of control, P = 0.23; Figs. 3A, B). When gap junction activity was assessed in cells transfected with AS-Cx43 oligonucleotide, decreased number of dye-coupled cell layers was observed (2.4 ± 0.38 vs. 4.1 ± 0.10, P = 0.012, n = 6; Figs. 4A, B). Cells transfected with random oligonucleotide showed significantly increased dye-coupled cell layers (4.6 ± 0.23 vs. 2.4 ± 0.38, P < 0.05, n = 6).

FIGURE 1. Effect of high glucose on Cx43 protein expression and apoptosis in RMECs. (A) A representative Western blot shows significant reduction in Cx43 protein level in cells grown in high-glucose medium compared to cells grown in normal medium. A bar graph indicates decreased Cx43 protein level in cells grown in high-glucose medium compared to cells grown in normal medium. Data were expressed as mean ± SD. *P < 0.05, n = 9. (B) Analysis of genomic DNA fragmentation in RMECs. Increased signal intensity in DNA laddering was observed in cells grown in high-glucose medium compared to those of cells grown in normal medium. (C) A bar graph shows approximately 50% increase in DNA laddering in cells grown in high-glucose medium. (D) An increased number of apoptotic cells was detected by differential staining. (E) An increased number of TUNEL+ cells was detected in cells grown in high-glucose medium. Data are expressed mean ± SD; *P < 0.05, n = 5.
oligonucleotide had no significant effect on dye-coupled cell layers compared to untransfected cells (4.3 ± 0.28; Figs. 4A, 4B). The concentration of Cx43 antisense oligonucleotides used in this study (0.1 μM) was designed to achieve approximately 50% downregulation of Cx43 localization, similar to the level exhibited by high-glucose cells. A strong correlation was observed between Cx43 protein level and the number of dye-coupled cell layers in normal cells, cells transfected with AS-Cx43 oligonucleotides or cells transfected with random oligonucleotides (r = .94). In addition, cells transfected with AS-Cx43 oligonucleotides not only exhibited reduced number of Cx43 punctate ‘dots’ (66% ± 8% of control, P < 0.001, n = 5), but that the number of dots was similar to those of cells grown in high-glucose medium (56% ± 14% of control, P = 0.001, n = 5; Figs. 2A, 2B). Overall, these results indicate that inhibition of Cx43 expression similar to the level exhibited by cells grown in high-glucose results in decreased gap junction intercellular communication activity.

**Effect of GJIC Inhibition on Microvascular Endothelial Cell Apoptosis**

β-GA-induced apoptosis, as assessed by TUNEL staining in both RMECs and RRECs in a concentration dependent manner. RMECs exposed to 50 μM or 100 μM β-GA exhibited increased apoptosis compared to control cells (185% ± 15% of control, P = 0.02 and 532% ± 35% of control, P = 0.004, respectively;
Fig. 5). Cell cultures treated with GZ, an inactive analog of the inhibitor, showed essentially no difference in apoptosis compared to cells grown in normal medium. RRECs grown in parallel exhibited similar results compared to RMECs. RRECs exposed to 50 μM H9262M or 100 μM H9252-GA exhibited increased apoptosis compared to control cells (237% ± 21% of control, P = 0.002 and 725% ± 64% of control, P = 0.0001, respectively), whereas β-GZ had practically no effect on apoptosis (Fig. 5).

The total number of dye-coupled cells on either side of the scrape line was clearly less in HG medium compared with normal medium (2.8 ± 1.2 vs. 5.7 ± 1.1, respectively). Cells grown in normal condition and exposed to 50 μM or 100 μM β-GA showed significantly reduced dye transfer compared to cells grown in normal medium (1.9 ± 0.5 vs. 5.7 ± 1.1 and 0.6 ± 0.2 vs. 5.7 ± 1.1, respectively). Cells grown in normal medium and exposed to β-GZ showed no effect compared to cells grown in normal medium (5.5 ± 1.0 vs. 5.7 ± 1.1).

**Increased Apoptosis in RMECs after Reduced Cell-cell Communication**

To determine the effect of reduced cell-cell communication on endothelial cell survival, Cx43-AS oligo-transfected RMECs that showed reduced dye transfer were assessed for apoptosis. DNA laddering assay showed a significant increase in the DNA band intensity in cells transfected with AS-Cx43 oligonucleotides compared to untransfected cells or cells transfected with random oligonucleotides. Fluorescent dye was detected in one to three layers in either side of the scrape line in cells transfected with AS-Cx43 oligonucleotides compared to four to six layers in the untransfected cells or cells transfected with random oligonucleotides indicating reduced GJIC activity in cells transfected with AS-Cx43 oligonucleotides. Magnification ×100. (B) A bar graph shows significant reduction in the number of dye-coupled cells in the high-glucose cells and AS-Cx43 oligonucleotide transfected cells. Rand, random. Data are presented as mean ± SD, *P < 0.05; n = 6.

**FIGURE 5.** β-GA, an inhibitor of GJIC, induced apoptosis in cultured retinal endothelial cells and microvascular endothelial cells. Cells were treated with β-GA (50 μM or 100 μM) or its inactive analogue (GZ, 50 μM) for 16 hours before analysis. Cells undergoing apoptosis were identified by TUNEL labeling. A significant increase in the number of TUNEL positive cells was observed when cells were exposed to 50 μM or 100 μM β-GA, whereas β-GZ showed no effect on apoptosis. Bars and ± error bars represent mean ± SD.
pared to the untransfected cells or cells transfected with random oligonucleotides (137.9 ± 16% of control, P = 0.003; 127.6% ± 17% of random control, Figs. 6A, B). Using differential staining assay we observed that cells transfected with AS-Cx43 exhibited a significant increase in the number of apoptotic cells compared to those of untransfected control cells or cells transfected with random oligonucleotides. (B) Graphical illustration shows significant increase in DNA fragmentation in cells transfected with AS-Cx43 oligonucleotides compared to those of untransfected control cells or cells transfected with random oligonucleotides. Data are presented as mean ± SD, *P < 0.05; n = 6.

FIGURE 6. Analysis of genomic DNA fragmentation in microvascular endothelial cells. (A) Total DNA from untransfected control cells, cells transfected with AS-Cx43 oligonucleotides or random (Ran) oligonucleotides was assessed for fragmentation by agarose gel electrophoresis and ethidium bromide staining. The relative intensity of DNA ladder was increased in cells with reduced Cx43 protein expression compared to those of untransfected control cells or cells transfected with random oligonucleotides. (B) Graphical illustration shows significant increase in DNA fragmentation in cells transfected with AS-Cx43 oligonucleotides compared to those of untransfected control cells or cells transfected with random oligonucleotides. Data are presented as mean ± SD, *P < 0.05; n = 6.

FIGURE 7. Representative photomicrographs of RMECs with differential staining that show transfection with AS-Cx43 oligonucleotides induces accelerated apoptosis: (A) untransfected normal cells, (B) AS-Cx43 oligonucleotides transfected cells, and (C) cells transfected with random oligonucleotides. Magnification ×100. (D) A bar graph shows cumulative data obtained from five experiments. Arrows point to apoptotic cells. Data are presented as mean ± SD, *P < 0.05; n = 5.

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DISCUSSION

Prior studies from our laboratory have indicated that Cx43 expression and cell-cell communication are reduced in RMECs and retinal pericytes grown in high-glucose condition.8,9 However, the consequence of such changes on cell survival has been unclear. In this study, we have determined that high glucose-induced downregulation of Cx43 expression and reduced cell-cell communication increases apoptosis in RMECs. Additionally, using AS-Cx43 oligonucleotides, we have determined the consequence of direct inhibition of Cx43 expression and reduced GJIC. Specific downregulation of Cx43 expression and reduced GJIC is sufficient to induce apoptosis in these cells. In line with our observation, other studies have reported increased apoptosis in different cell types with reduced connexin expression.13,16–19 To our knowledge this is the first study that shows specific downregulation of Cx43 expression and reduced GJIC may be one of the mechanisms by which high glucose contributes to the development of apoptosis in RMECs, and that high glucose-induced Cx43 downregulation is sufficient to induce apoptosis.

The mechanisms responsible for inducing retinal vascular cell apoptosis in diabetes are not fully identified and could conceivably be manifested at the level of gap junction activity. When cells are connected via gap junction channels they can exchange ions and small metabolites and form a cell-cell communication network that contributes to maintenance and reg-

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have reported that Cx43 protein is the most abundant of the three Cxs (Cx37, Cx40, and Cx43) in the RMECs, and that high glucose alters Cx43 expression the most. Currently it is unknown why high glucose would affect Cx43 expression more than the other Cxs. Interestingly, we have observed that heterogenous gap junctions composed of Cx43/Cx40 appears to be downregulated by high glucose, suggesting complex interactions between Cx40 and Cx43. Also, the reduction in Cx43 may be due to decreased synthesis, increased degradation, or both. While an association between cell-to-cell communication and cell survival has been reported in different cell types, this study demonstrates that cell survival can be compromised as a consequence of high glucose–induced altered cell-to-cell communication in microvascular endothelial cells and retinal endothelial cells. The current finding together with our previous reports and other investigators’ report clearly shows that high glucose–induced apoptosis is at least in part associated with decreased Cx43 expression in RMECs.

Vascular endothelial cells and pericytes communicate with each other through a complex interactive network in the retinal microvessels. Since connexin channels are present on endothelial cells and pericytes, they likely play a regulatory role by facilitating the passage of ions and secondary messengers such as cAMP, Ca2+ required for cell survival, growth, proliferation, and homeostasis. Recent studies have indicated that cell survival signals are transduced by Cx43 channels, and that Cx43 channels have 300-fold higher selectivity for ATP compared to other connexin channels. Therefore, high glucose–induced inhibition of Cx43 expression can influence the development of cell apoptosis and eventual disruption of vascular homeostasis in diabetic retinopathy.

Currently it is unknown how high glucose initiates apoptosis in microvascular endothelial cells. The finding that high glucose reduces Cx43 expression and inhibits GJIC that is linked to microvascular endothelial cell death may offer, at least in part, a potential mechanism by which high glucose triggers apoptosis. In this study, we observed that downregulation of Cx43 expression with Cx43 antisense oligos and inhibition of GJIC with known gap junction inhibitor leads to apoptosis. In addition, as suggested by Fernandes et al., since PKC activity is increased under hyperglycemic condition, it is possible that high glucose–induced phosphorylation of Cx43 in RRECs could facilitate Cx43 degradation by a proteasome-dependent mechanism, resulting in reduced GJIC. Taken together, the findings from these studies indicate that hyperglycemia-induced downregulation of Cx43 expression and GJIC could play an important role in promoting apoptosis.

Although the occurrence of apoptosis in the diabetic retina is a well-established phenomenon, mechanism(s) by which hyperglycemia induces apoptosis remain unclear. Since high glucose inhibits cell-cell communication, it may restrict not only the “inward” flow of cell survival signals into the cells but also restrict the “outward” flow of toxic elements such as glutamate, which could contribute to glutamate toxicity. Loss of connexin function preferentially degrades occludin-based tight junctions and could promote vascular permeability in the diabetic retina. Additionally, impairment in transport of ions such as Ca2+ may retard cell growth. Our findings indicate that cell-cell communication is at least in part necessary for cell survival, that high glucose-induced disruption in cell-cell communication may be an early trigger for inducing apoptosis, and that high glucose–induced inhibition of this interaction could promote cell loss of retinal microvascular cells, a prominent characteristic of diabetic retinopathy. It remains to be determined whether reduced Cx43 expression...
and GJIC activity promotes apoptosis in the vascular cells of the diabetic retina. Further studies are necessary to elucidate the effect of reduced cell-cell communication in diabetic retinopathy.

References