

Reduced Connexin 43 Expression and Its Effect on the Development of Vascular Lesions in Retinas of Diabetic Mice

Michael W. Bobbie,^{1,2} Sumon Roy,^{1,2} Kyle Trudeau,^{1,2} Stephanie J. Munger,³ Alexander M. Simon,³ and Sayon Roy^{1,2}

PURPOSE. To examine whether diabetes-induced connexin 43 downregulation promotes retinal vascular lesions characteristic of diabetic retinopathy (DR).

METHODS. Two animal models, streptozotocin-induced diabetic mice and Cx43 heterozygous knockout (Cx43[±]) mice, were studied to directly assess whether diabetes reduces the expression of retinal Cx43, which, in turn, contributes to retinal vascular cell loss by apoptosis. Retinal Cx43 protein levels were assessed in nondiabetic control mice, diabetic mice, and Cx43[±] mice by Western blot analysis, and Cx43 localization and distribution in the retinal vascular cells were studied by immunostaining of retinal trypsin digests (RTDs). In parallel, RTDs were stained with hematoxylin and periodic acid Schiff to determine pericyte loss (PL) and acellular capillaries (AC), and TUNEL assays were performed to determine retinal vascular cell apoptosis.

RESULTS. Western blot analysis indicated significant reductions in retinal Cx43 protein levels in diabetic mice and Cx43[±] mice compared with those of nondiabetic mice. Similarly, a significant reduction in Cx43 immunostaining was observed in the retinal capillaries of diabetic mice and Cx43[±] mice compared with those of control mice. Both diabetic and age-matched Cx43[±] mice exhibited increased amount of PL, AC, and TUNEL-positive cells compared with control mice.

CONCLUSIONS. Diabetes-induced inhibition of Cx43 expression contributes to vascular cell apoptosis in retinas of diabetic mice. This suggests that reduced Cx43 expression plays a critical role in the development of AC and PL associated with DR. (*Invest Ophthalmol Vis Sci.* 2010;51:3758–3763) DOI: 10.1167/iovs.09-4489

Gap junctions are membrane channels that allow the transfer of ions and small molecules <1 kDa¹ between adjacent cells for the maintenance of vascular homeostasis.^{2,3} Each of these channels is composed of two connexin hemichannels that couple with one another to form a functional gap junction channel.⁴ The presence of gap junctions in the retina and an

extensive cell-to-cell coupling within the retinal microvessels have been well documented.⁵ Importantly, an abundant expression of Cx43 in the retinal endothelial cells and pericytes⁶ suggests substantial gap junction coupling in the retinal vascular cells. Studies investigating gap junctions between pericytes and endothelial cells in culture identified the junctional transfer of small molecules.⁷ Examination of human retinal capillaries has revealed that the basement membrane interposed between endothelial cells and pericytes is absent at specific points to permit cell membrane contacts⁸ that could facilitate gap junction activity between these cells.

Abnormal connexin expression in diabetes is associated with the development of various complications involving the skin,⁹ kidneys,¹⁰ bladder,¹¹ perineurium,¹² lens,¹³ heart,¹⁴ and other tissues; however, its role in the development of vascular lesions in the diabetic retina remains unknown. Although hyperglycemia is known to induce apoptosis and trigger retinal vascular cell loss, a hallmark of background DR, the mechanisms underlying hyperglycemia-induced apoptosis are not completely understood. Increasing evidence indicates that compromised gap junction activity can have serious consequences, including the initiation of apoptosis and the breakdown of vascular homeostasis. Gap junction-mediated exchange of small molecules, such as nucleotides, cAMP, IP₃, and Ca²⁺, plays a critical role in cell function and viability.¹⁵ Studies investigating the role of reduced gap junction intercellular communication (GJIC) activity on cell survival or apoptosis have reported that the downregulation of Cx43 expression increases apoptosis in rat ventricular myocytes¹⁶ and microvascular endothelial cells.¹⁷ Increased apoptosis was reported in Cx43[±] mice in which astrocytic gap junctional communication was decreased.¹⁸ Further studies with Cre mice with floxed Cx43 lacking the Cx43 gene in astrocytes showed increased apoptosis in cells representing penumbral lesions.¹⁹ Similarly, osteoblast cells derived from Cx43-null mice exhibited increased apoptosis.²⁰ Taken together, these studies suggest that an alteration in Cx43 expression profoundly affects cell survival.

Our previous studies have shown that high glucose downregulates Cx43 expression in microvascular endothelial cells and retinal pericytes, with concomitant reductions in GJIC activity.^{21,22} Recently, we have shown that high glucose-induced downregulation of Cx43 expression is an early trigger for inducing apoptosis in retinal endothelial cells.¹⁷ In line with our findings, other reports have indicated high glucose-induced Cx43 downregulation in retinal endothelial cells in vitro.²³ Studies investigating pericytes isolated from diabetic rat retinas have shown a dramatic reduction in cell-to-cell coupling.⁵ Although these studies demonstrate that high glucose-induced downregulation of Cx43 expression and GJIC promotes apoptosis in vitro, it is unknown whether diabetes promotes similar changes in vivo in the retina, contributing to

From the Departments of ¹Medicine and ²Ophthalmology, Boston University School of Medicine, Boston, Massachusetts; and the ³Department of Physiology, University of Arizona, Tucson, Arizona.

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Corresponding author: Sayon Roy, 650 Albany Street, Departments of Medicine and Ophthalmology, Boston University School of Medicine, Boston, MA 02118; sayon@bu.edu.

retinal vascular cell loss and the breakdown of retinal vascular homeostasis associated with DR. In this study, we investigated whether diabetes-induced downregulation of Cx43 expression promotes characteristic microvascular cell loss in two animal models, the streptozotocin-induced diabetic mouse and the Cx43[±] mouse.

METHODS

Animals

All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Cx43[±] mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained on a C57Bl6 strain background. Genotypes were determined by polymerase chain reaction (PCR) at weaning using tail tip DNA, and then again at kill. PCR reactions were performed with a PCR enzyme blend (Advantage 2 Polymerase; Clontech, Mountain View, CA) and included the following primers: primer 1, 5'-CCCACTCTCACCTATGTCTCC-3'; primer 2, 5'-ACTTTTGCCGCCTAGCTATCCC-3'; primer 3, 5'-ATAGCGTTGGCTACCCGTGA-3'. Primers 1 and 2 amplify an approximately 500-bp fragment from the Cx43 wild-type allele. Primers 2 and 3 amplify an approximately 900-bp fragment from the Cx43 knockout allele. In this study, we used Cx43[±] mice and wild-type littermates randomly selected to represent the normal and the diabetic group (*n* = 6 per group). The Cx43^{-/-} genotype was not used because it is lethal.²⁴

Diabetes was induced by intraperitoneal injection of streptozotocin at a dose of 50 mg/kg body weight on 5 consecutive days. NPH insulin (0.1–0.4 U) was administered subcutaneously as needed to achieve slow body weight gain without preventing hyperglycemia and glycosuria. Sterile diluent (Eli Lilly, Indianapolis, IN) was used to dilute insulin before injection. Body weight and glucose levels were monitored throughout the study in all mice. Animals approximately 24 weeks old were killed by the end of 12 weeks of diabetes. Both eyes were enucleated, and retinas were isolated; half of one retina of each animal was used for protein isolation, and the other half of that retina along with the contralateral retina were used for retinal trypsin digest (RTD) preparation. At the time of kill, HbA1c levels were measured for all mice to verify hyperglycemic status in the diabetic mice compared with normal mice and Cx43[±] mice.

Western Blot Analysis

Western Blot analysis was performed to determine the relative levels of Cx43 protein in the mouse retinas from each group. Briefly, retinas were dissected from the enucleated eyes and placed in a buffer containing 25 mM Tris (pH 7.4), 1 mM EDTA, and 0.1% Triton X-100. Retinal samples were homogenized, and protein was isolated as previously described.²⁵ Bicinchoninic acid assay (Pierce Chemical, Rockford, IL) was used to determine total protein concentrations. Western blot analysis was performed with 25 µg protein/lane; after electrophoresis, the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) using a semidry apparatus according to Towbin's procedure (<http://diabetes.diabetesjournals.org/cgi/content/full/55/1/86-R22>).²⁶ The membranes were blocked with 5% nonfat dry milk for 2 hours and then exposed to mouse monoclonal anti-Cx43 antibody (Millipore, Billerica, MA) solution (1:1000) overnight at 4°C. Blots were washed with Tris-buffered saline containing 0.1% Tween-20 and then incubated with rabbit anti-mouse IgG secondary antibody (Sigma, St. Louis, MO) solution (1:15,000) for 1 hour. The membranes were again washed as described and then exposed (Immun-Star Chemiluminescent Protein Detection System; Bio-Rad) to detect the protein signals on x-ray film (Fujifilm, Tokyo, Japan). Protein loading in the gels was confirmed by Ponceau-S staining and β-actin antibody binding. The densitometric values of the Ponceau-S-stained membranes and β-actin signals were used to correct for the Western blot signals. The Western blot membranes exposed to Cx43 antibody were stripped with a buffer containing 62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM 2-mercapto-

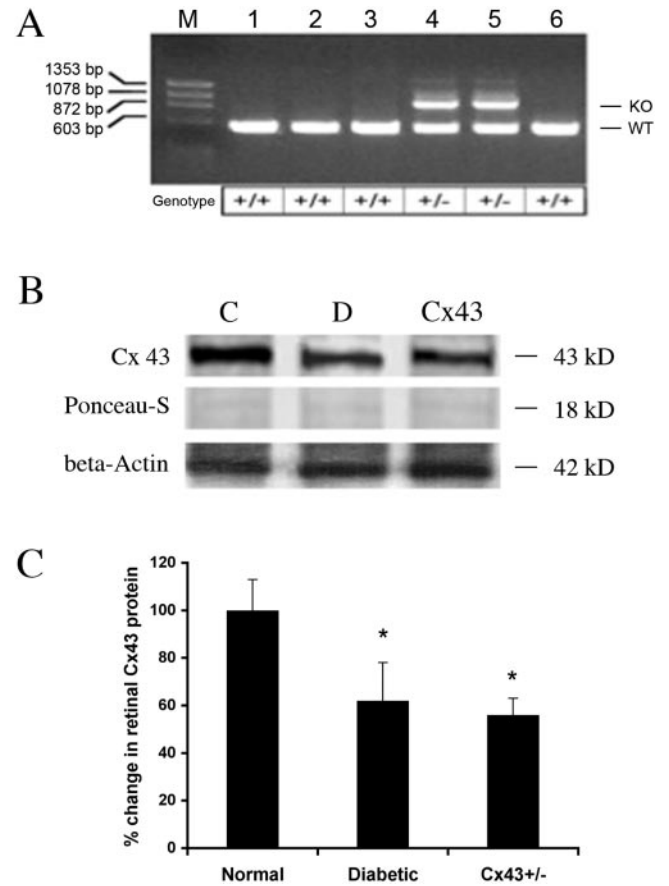


FIGURE 1. (A) PCR analysis with mice tail tip DNA showing genotypes of Cx43 heterozygous (\pm) and wild-type mice. The wild-type allele is represented by a band at 500 bp, whereas the disrupted allele shows a band at 900 bp in the ethidium bromide-stained gel. (B) Western blot analysis shows relative amounts of Cx43 protein in retinas of control mouse eyes, diabetic mouse eyes, and Cx43[±] mouse eyes. Corresponding Ponceau-S and β -actin Western blot signal to the stripped membrane shows equal protein loading (*lower*). (C) Retinal Cx43 protein levels are reduced in diabetic and Cx43[±] mice compared with control mice. **P* < 0.01.

ethanol and were reused with β -actin antibody (A5060; Sigma; 1:2500). After washing, the membranes were incubated with secondary antibody as described for Cx43 antibody detection. Densitometric analysis of the Western blot signals was performed at nonsaturating exposures and analyzed using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).

Retinal Trypsin Digestion

To analyze the retinal vasculature for acellular capillaries (ACs) and pericyte loss (PL), the RTD technique was performed as described,²⁷ with slight modifications. Briefly, the retinas were dissected from the enucleated eyes and subjected to 3% trypsin digestion (Becton-Dickinson, San Jose, CA) at 37°C for approximately 3 hours with gentle shaking. Under a dissecting microscope, the nonvascular mass of the retina was removed from the vascular network, which was then mounted on a silane-coated slide.

Immunostaining for Cx43 and Analysis of RTDs

To study the distribution of Cx43 gap junctions in the retinal capillaries, immunostaining was performed in RTD preparations with Cx43 antibodies. Briefly, the RTDs were blocked for 15 minutes in 2% BSA to prevent nonspecific antibody binding, and then they were incubated

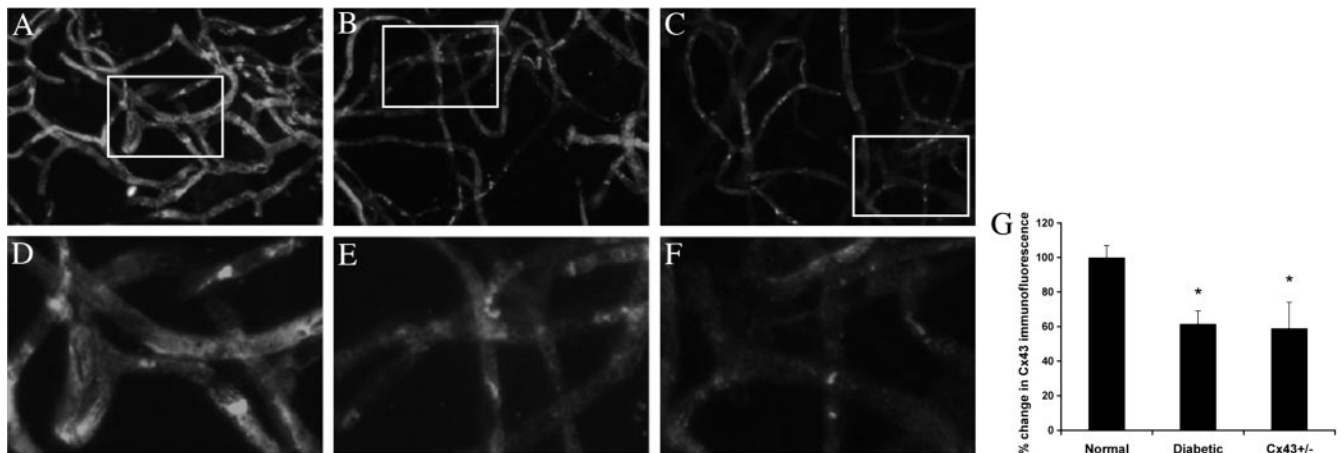


FIGURE 2. Representative images showing Cx43 immunostaining in retinal vessels of (A) control mice, (B) diabetic mice, and (C) Cx43^{-/-} mice. (D–F) Images showing enlarged views of insets. (D) Control mice. (E) Diabetic mice. (F) Cx43^{+/-} mice. (G) Cx43 immunostaining was significantly reduced in the retinal vessels of diabetic and Cx43^{+/-} mice compared with control mice. * $P < 0.01$.

overnight at 4°C in a moist chamber using mouse monoclonal anti-Cx43 antibody (1:100 dilution with 2% BSA; Millipore). After the overnight incubation, the RTDs were washed in PBS and incubated at room temperature with rhodamine-conjugated rabbit anti-mouse IgG secondary antibody (1:100 dilution with 2% BSA) for 1 hour (Jackson ImmunoResearch, West Grove, PA). After three PBS washes, RTDs were mounted in reagent (SlowFade; Molecular Probes, Eugene, OR), digital images were captured, and Cx43 immunostaining was quantified from 10 random representative fields from each RTD.

Detection of Acellular Capillaries and Pericyte Loss

To analyze AC and PL, RTDs representing the three groups of mice were stained with periodic acid-Schiff and Harris hematoxylin (Sigma-Aldrich, St. Louis, MO). RTD slides were immersed in 0.5% PAS (Sigma-Aldrich) for 10 minutes, rinsed in dH₂O, and exposed to Schiff's reagent (Electron Microscopy Sciences, Hatfield, PA). Next, the slides were rinsed in dH₂O and immersed in Harris hematoxylin (Sigma-Aldrich) for 20 seconds. After rinsing in dH₂O, the slides were subjected to dehydration through an ethanol gradient and clearing in xylene and then were mounted in mounting medium (Permount; Fisher Scientific, Pittsburgh, PA). Ten representative 36 $\mu\text{m} \times 27 \mu\text{m}$ images were photographed using a digital camera (DS-Fi1; Nikon, Tokyo, Japan) connected to a computer, and the images were analyzed for AC and PL. Apoptotic pericytes were identified as PL (pericyte ghost) based on prominent histologic characteristics, including basement membrane protrusion as empty shell. Capillaries devoid of both pericytes and endothelial cells were considered as AC. Counts were also scored by a second independent examiner in a masked manner. The data represent the average of both scores.

Terminal dUTP Nick-End Labeling

To determine apoptosis, TUNEL assay was performed on RTD slides representing all three experimental groups with a commercial kit (ApoTag In Situ Apoptosis Detection; Chemicon, Temecula, CA) according to the manufacturer's instructions. Briefly, RTDs were permeated with a precooled mixture of a 2:1 ratio of ethanol/acetic acid. After two washes in 1 \times PBS, slides were incubated with equilibration buffer and incubated with TdT enzyme in a moist chamber at 37°C for 1 hour. Slides were then washed with 1 \times PBS and incubated with anti-digoxigenin peroxidase. Finally, the slides were washed in 1 \times PBS and mounted using reagent (SlowFade; Molecular Probes). Images from 10 random fields representing each RTD were captured using a digital microscope (DS-Fi1; Nikon) and recorded for analysis.

Statistical Analysis

Data are presented as mean \pm SD. Values of the control groups were normalized to 100%, and values from all other groups were expressed as percentages of control. Statistical analysis was performed using the normalized values. Comparisons between groups were performed using ANOVA followed by the Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Glycemic Levels, Body Weights, and Genotyping of Control and Experimental Animals

Throughout the study the fasting blood glucose measurements showed significant increases in blood glucose levels in the diabetic mice compared with those of the control mice (284.9 \pm 108.8 mg/dL vs. 133.3 \pm 27.6 mg/dL; $P < 0.001$) or Cx43^{+/-} mice (284.9 \pm 108.8 mg/dL vs. 139.3 \pm 13.5 mg/dL; $P < 0.001$). Blood glucose levels did not show a significant difference between the control and the Cx43^{+/-} group ($P = 0.602$). There was no significant difference in body weight among the three study groups. Average body weights were 24.0 \pm 4.0 g for the control mice, 23.3 \pm 3.7 g for the diabetic mice, and 23.6 \pm 4.1 g for the Cx43^{+/-} mice. HbA1c levels were significantly increased in diabetic mice (10.1% \pm 1.7%) compared with nondiabetic control (4.2% \pm 1.1%) and Cx43^{+/-} mice (4.6% \pm 1.0%). Mouse genotypes were determined by PCR at weaning using tail tip DNA and then again at kill. PCR-amplified fragments representing wild-type control mice showed one band; Cx43^{+/-} mice showed two bands, confirming the heterozygous knockout genotype (Fig. 1A).

Diabetes Downregulates Cx43 Expression in Retinas of Diabetic Mice

Western blot analysis showed that in the diabetic mice, retinal Cx43 protein levels were significantly lower than those in control mice (60.7% \pm 13.1% of control; $P < 0.01$) (Figs. 1B, 1C). As expected, the Cx43^{+/-} mice showed a decrease in retinal Cx43 protein levels compared with those of control mice (53.1% \pm 7.5% of control, $P < 0.001$). Similarly, Cx43 immunostaining was significantly reduced in RTDs of diabetic mice compared with those of control mice (61.5% \pm 7.5% of control, $P < 0.001$; Fig. 2). As expected, Cx43 immunostaining of RTDs of Cx43^{+/-} mice exhibited a significant decrease com-

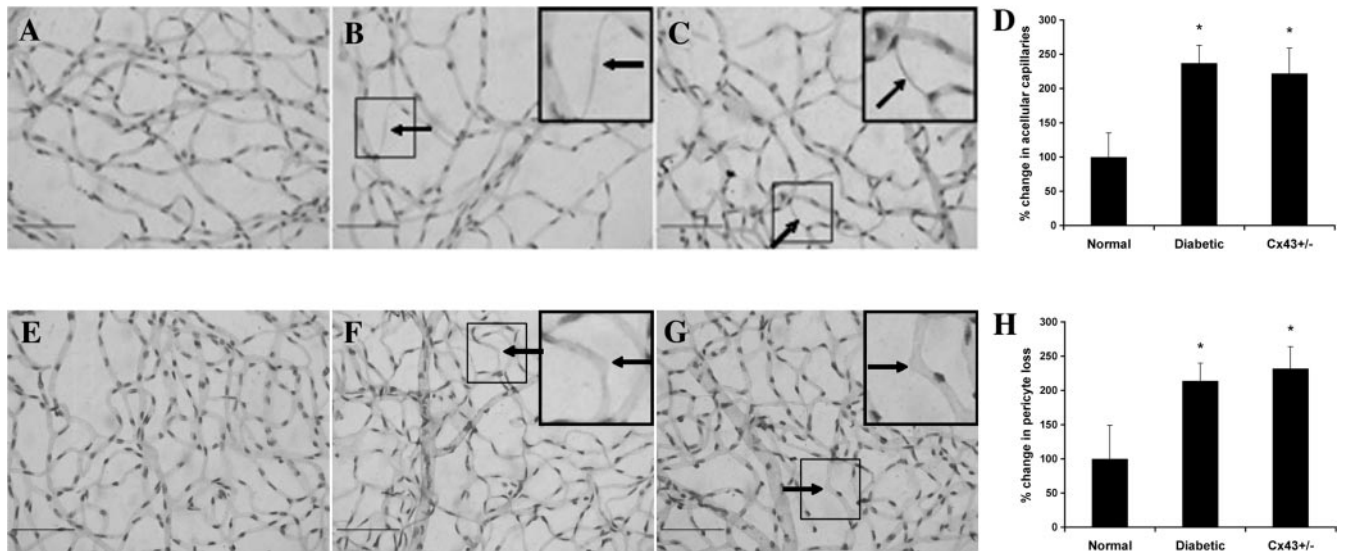


FIGURE 3. Representative images of retinal capillary network stained with hematoxylin and periodic acid-Schiff in (A, E) control mice, (B, F) diabetic mice, and (C, G) Cx43[±] mice. Scale bars, 5 μm. (A–C, arrows) ACs in the RTDs of capillary networks. *Insets*: enlarged images showing ACs. (D) Percentage change in the number of ACs. The average number of ACs in retinas of diabetic and Cx43[±] mice was significantly increased compared with control. **P* < 0.001. (E–G, arrows) PL in the RTDs of capillary networks. *Insets*: enlarged images showing PL. (H) Percentage change in the amount of PL. The average amount of PL in the retinas of diabetic and Cx43[±] mice was significantly increased compared with control. **P* < 0.001.

pared with that of control mice (59.0% ± 15% of control, *P* < 0.01).

Increased Numbers of Acellular Capillaries and Pericyte Loss in Retinas of Mice with Reduced Cx43 Protein Expression or Diabetes

The numbers of AC and PL in retinas of diabetic mice were significantly increased compared with those of control (237% ± 26% of control [*P* < 0.0001] and 214% ± 26% of control [*P* < 0.001], respectively; Fig. 3). In the retinas of Cx43[±] mice, significant increases in AC and PL were observed and were similar to those in the diabetic mice (222% ± 37% of control

[*P* < 0.001] and 232% ± 32% of control [*P* < 0.001], respectively).

Accelerated Cell Death in Retinal Vasculatures of Cx43[±] and Diabetic Mice

The number of TUNEL-positive cells in retinal capillaries of diabetic mice was significantly increased compared with that of control mice (218% ± 23% of control, *P* = 0.042; Fig. 4). Similarly, in the retinal capillaries of Cx43[±] mice, the number of apoptotic cells was significantly increased compared with that of control mice (251% ± 19% of control, *P* = 0.043).

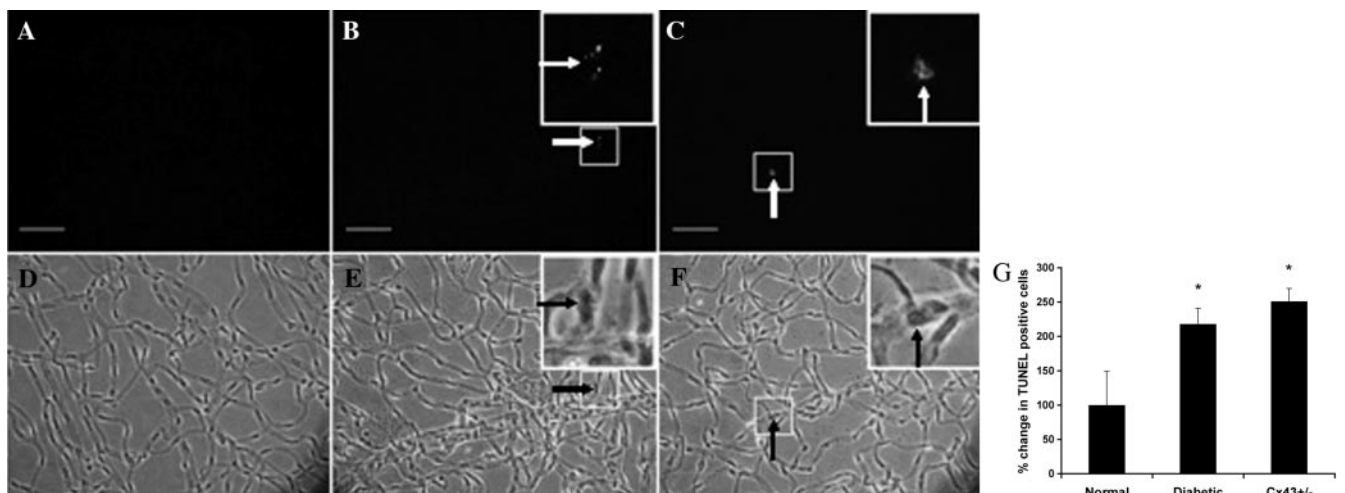


FIGURE 4. Representative images of capillary networks showing TUNEL-positive cells in (A) control mice, (B) diabetic mice, and (C) Cx43[±] mice. *Lower*: corresponding bright-field images of the capillary network in (D) control mice, (E) diabetic mice, and (F) Cx43[±] mice. Scale bar, 5 μm. *White arrows*: TUNEL-positive signals. *Black arrows*: corresponding apoptotic cells in the bright-field images of the retinal capillary networks. Increased number of TUNEL-positive cells was present in both experimental groups compared with the control group. (G) Percentage change in the number of TUNEL-positive cells in the retinal capillaries. The number of TUNEL-positive cells increased significantly in the retinas of diabetic and Cx43[±] mice compared with those of control. **P* < 0.05.

DISCUSSION

This is the first report indicating that diabetes reduces Cx43 expression in the retina and that a reduction in Cx43 expression promotes the development of AC and PL similar to the development seen in DR. Increases in the numbers of AC and PL in the diabetic mice were strikingly similar to those observed in the Cx43[±] mice, suggesting that a 50% decrease in Cx43 expression is sufficient to induce the development of AC and PL, as seen in diabetes. Data from TUNEL assay confirmed that the retinal vascular cell loss observed in the Cx43[±] mice resulted from apoptosis, as seen in the retinas of diabetic mice.

The current findings are supported by previous *in vitro* studies showing that high glucose reduces Cx43 gene expression in retinal endothelial cells and pericytes.^{21,22} More recently, Cx43 downregulation and a concomitant reduction in GJIC activity was shown to be sufficient to induce apoptosis.¹⁷ In line with our previous findings, a study showed that high glucose-induced Cx43 downregulation can occur through increased phosphorylation and elevated proteasomal degradation of Cx43.²³ Additionally, cell-to-cell coupling in freshly isolated retinal pericytes was shown to be reduced in diabetic rats.⁵ Although these studies provide valuable information related to reduced Cx43 expression, decreased GJIC activity, and its damaging effects, the current findings provide evidence that diabetes-induced Cx43 downregulation can promote vascular cell loss in the retina. This implicates hyperglycemia-induced inhibition of Cx43 expression as a potential mechanism contributing to the development of AC and PL in diabetes.

Although altered Cx43 levels play a significant role in the development of complications in vascular diseases, their involvement in the pathogenesis of DR is unclear. In prehypertensive rats, the aortic endothelium exhibits decreased Cx43 expression and contributes to accelerated vascular cell wall remodeling.²⁸ In late stages of human coronary atherosclerosis, Cx43 is downregulated, and this decline parallels the accumulation of extracellular matrix material.²⁹ Interestingly, we have observed that high glucose-induced overexpression of extracellular matrix components regulates Cx43 expression.³⁰ Although Cx43 is known to be widely expressed in the normal retina, its involvement in the development of vascular lesions in the diabetic retina has been unclear. In diabetes, differential expression of Cx43 has been reported in several organs and cell types: decreased Cx43 expression in delayed wound healing in the diabetic skin,⁹ marked decreases in Cx43 in endothelial cells related to compromised renal blood flow in diabetes,¹⁰ and time-dependent and cell-specific decreases in Cx43 in urinary bladder dysfunction in diabetes.¹¹ Taken together, these reports and our current findings indicate that decreased Cx43 expression plays an important role in various pathologic conditions, including retinal vascular cell loss associated with DR.

It is unknown whether reduced connexin expression in the retina may affect other cellular events associated with the development of DR. The docking of connexin hemichannels between adjacent cells is facilitated by cadherin proteins.³¹ In nonproliferative DR, cadherin-5 expression in the retina is reduced,³² which may compromise interactions between cadherin and the cytoplasmic loop domain of Cx43.³³ Such altered interactions could compromise barrier characteristics in the retinal vessels. Additionally, because blood-retinal barrier (BRB) breakdown in diabetes involves the decreased expression of tight junction protein ZO-1,³⁴ which can alter Cx43 function³⁵ through interaction with the COOH-terminal of Cx43,³⁶ it is possible that in diabetes, BRB breakdown involves both tight junctions and gap junctions.

Although hyperglycemia is known to trigger apoptosis and to play a prominent role in retinal vascular cell loss, it is unclear

through which mechanism hyperglycemia induces apoptosis. Increasing evidence suggests that cell death or cell survival modulators exchanged through gap junctions influence apoptotic events. However, despite several studies investigating the role of connexin in mediating apoptosis, the findings remain inconclusive largely because of the complexity involving the exchange of both cell death and cell survival signals that can pass through these channels. Additionally, the exchange of these modulators could be influenced by the cell type, the cellular context, and the impact connexin proteins have beyond their channel function on the cell death process. For example, connexin hemichannel-mediated alteration in signal transduction can affect mitochondrial functioning and could contribute to cell death. Taken together, these findings suggest that under high glucose or hyperglycemic conditions, a build-up of proapoptotic messengers including IP₃, glutamate, and NAD⁺, among others, is accelerated because their ability to leave the cell is thwarted by reduced connexin channels. Our findings provide new evidence that diabetes-induced downregulation of Cx43 may disturb vascular homeostasis and that it promotes apoptosis in DR. Strategies to protect retinal vascular cells by regulating cell-to-cell communication in diabetes may have therapeutic value.

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