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Association of reduced Connexin 43 expression with retinal vascular lesions in human diabetic retinopathy

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ABSTRACT

Connexin 43 (Cx43) downregulation promotes apoptosis in retinal vascular cells of diabetic animal models; however, its relevance to human diabetic retinopathy has not been established. In this study, we investigated whether diabetes alters Cx43 expression and promotes retinal vascular lesions in human retinas. Diabetic human eyes (aged 64–94 years) and non-diabetic human eyes (aged 61–90 years) were analyzed in this study. Retinal protein samples and retinal capillary networks were assessed for Cx43 level by Western blot (WB) analysis and immunostaining. In parallel, retinal capillary networks were stained with hematoxylin and periodic acid Schiff to determine the extent of pericyte loss (PL) and acellular capillaries (AC) in these retinas. Cx43 protein expression was significantly reduced in the diabetic retinas compared to non-diabetic retinas as indicated by WB analysis (81 ± 11% of control). Additionally, a significant decrease in the number of Cx43 plaques per unit length of vessel was observed in the diabetic retinas compared to those of non-diabetic retinas (62 ± 10% of control; $p < 0.005$). Importantly, a strong inverse relationship was noted between Cx43 expression and the relative number of AC ($r = -0.89$; $p < 0.0005$), and between Cx43 expression and number of pericyte loss ($r = -0.88$; $p < 0.0005$). Overall, these results show that Cx43 expression is reduced in the human diabetic retinas and Cx43 reduction is associated with increased vascular cell death. These findings suggest that diabetes decreases retinal Cx43 expression and that the development of PL and AC is associated with reduced Cx43 expression in human diabetic retinopathy.

Gap junction intercellular communication (GJIC) plays a major role in the maintenance of vascular homeostasis by connecting the cytoplasm of two adjacent cells and allowing the passage of small molecules and ions between these cells. Gap junctions are present in cells of many tissues including the retina where Connexin 43 (Cx43) is particularly abundant. In vitro studies have shown that high glucose impairs GJIC activity in retinal endothelial cells (Fernandes et al., 2004), pericytes (Li et al., 2003), and microvascular endothelial cells (Sato et al., 2002). Changes in Cx43 expression and gap junction activity have also been described in the diabetic retina as well as other diabetic tissues, including skin (Wang et al., 2007), lens epithelium (Lin et al., 2007) and kidneys (Sawai et al., 2006), and has lead to a better understanding of the importance of Cx43-mediated GJIC activity in diabetes.

In diabetic retinopathy, apoptotic cell death of retinal microvascular cells is manifested as acellular capillaries and pericyte loss, hallmarks of early stages in the disease process. An increasing number of studies have established that downregulation of Cx43 expression and reduced GJIC can promote apoptosis in retinal vascular cells (Bobbie et al., 2010; Li and Roy, 2009; Tien et al., 2014). Such Cx43-mediated cell–cell communication is necessary for maintenance of retinal vascular homeostasis and plays a critical role in cell survival in the diabetic retina. Studies from our lab have shown that high glucose can influence cell survival by altering Cx43 expression and disrupting intracellular communication between endothelial cells, between endothelial cells and pericytes, and more recently, between pericytes and retinal Muller cells (Li and Roy, 2009; Li et al., 2003; Muto et al., 2014). In addition, our previous work using a Cx43 ± knockout mouse model indicated that diabetes-induced inhibition of Cx43 expression contributes to vascular cell apoptosis in retinas of diabetic mice (Bobbie et al.,

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2010). These studies suggest that reduced Cx43 expression plays a key role in the development of AC and PL associated with diabetic retinopathy.

Furthermore, chronic hyperglycemia in diabetes is associated with the development and progression of pathological changes in the retinal vasculature involving the breakdown of the blood retinal barrier (BRB). The endothelium regulates the BRB in the retinal capillaries at least in part through tight junctions (Dejana et al., 1995; Do carmo et al., 1998). Interestingly, we have observed that high-glucose induced downregulation of Cx43 and reduced GJIC can increase cell monolayer permeability by compromising tight junction proteins, in particular, ZO-1 and occludin (Tien et al., 2013). We have also demonstrated that in rats treated with intravitreal injections of Cx43-siRNA, selective downregulation of Cx43 expression induces vascular cell death and promotes vascular permeability in the retina (Tien et al., 2014). These findings suggest not only that impaired GJIC among retinal vascular cells under high-glucose conditions may trigger microvascular cell apoptosis but also that Cx43 downregulation may contribute to altered endothelium in retinal vasculature leading to BRB breakdown in diabetic retinopathy. Taken together, these studies indicate that Cx43 downregulation promotes vascular cell loss and increases vascular permeability in diabetic animals. However, it is unknown whether altered Cx43 expression and reduced GJIC promotes retinal vascular lesions in the human diabetic retina. In the current study, we examined whether diabetes alters Cx43 expression and promotes retinal vascular lesions in human retinas.

Donor eye samples were selected from a tissue collection at the University of Iowa. All experiments complied with the Declaration of Helsinki. Preferential criteria for inclusion in the study were specific to the stages of development in diabetic retinopathy, and the least possible chronic pathology other than diabetes. Criteria for exclusion were presence of other retinal or hematologic disease, uremia, and administration of chemotherapy or life support measures. Tissues from human donor eyes of 6 non-diabetic individuals (age 61–90 years; mean age 83 ± 11 years) and 6 diabetic individuals with a history of diabetic retinopathy (age 64–94 years; mean age 80 ± 10 years) were analyzed for assessment of Cx43 plaques, acellular capillaries, and pericyte loss. Table 1 reports the clinical characteristics of these donors. Posterior poles were fixed for 4 h in 4% paraformaldehyde. All samples were preserved within 9 h of death (average death to fixation time 6.6 h). Extramacular temporal retina was collected and used for morphological analysis. Retinas were studied for Cx43 protein levels from diabetic and non-diabetic subjects using WB analysis. In addition, retinal vasculature networks were isolated from the retinas using the trypsin digest technique, followed by Cx43 immunostaining and counterstaining with PAS/hematoxylin.

Eyes were processed as previously described (Roy et al., 1996) and retinal protein was subjected to WB analysis (Bobbie et al., 2010). Briefly, retinal samples were homogenized in lysis buffer containing 25 mM Tris (pH 7.4), 1 mM EDTA, and 0.1% Triton X-100. Bicinchoninic acid assay (Pierce Chemical, Rockford, IL) was used to determine the total protein concentrations, and protein samples were subjected to electrophoresis in polyacrylamide gel and transferred to polyvinyl difluoride (PVDF) membrane using a semidry apparatus. Membranes were subsequently incubated overnight at 4 °C with rabbit Cx43 (Cell Signaling, Danvers, MA) followed by anti-rabbit secondary antibody conjugated with alkaline phosphatase (1:3000) (Cell Signaling). Densitometric values were determined to ascertain differences in Cx43 protein levels.

To analyze the retinal vasculature for acellular capillaries and pericyte loss, the retinal trypsin digestion (RTD) technique was performed as described (Kuwabara and Cogan, 1960) with slight modifications. The retina was subjected to 3% trypsin digestion at 37 °C for approximately 2 h with gentle shaking. The nonvascular mass of the retina was removed from the vascular network under a dissecting microscope and was subsequently mounted on a silane-coated glass slide.

To study the distribution of Cx43 gap junctions in the retinal capillaries, immunostaining was performed in RTD preparations with Cx43 antibodies. The vascular networks were blocked in 2% bovine serum albumin and incubated overnight at 4 °C in mouse monoclonal anti–Cx43 antibody (Millipore, Billerica, MA), followed by incubation in rhodamine-conjugated rabbit anti–mouse IgG secondary antibody (Jackson Labs, West Grove, PA). The RTDs were washed with PBS. Digital images were captured, and the number of Cx43 plaques per unit of vessel length was quantified using ImageJ analysis.

After immunofluorescent imaging, the retinal vascular networks were counterstained with periodic acid-Schiff and hematoxylin to detect acellular capillaries and pericyte loss. RTD slides were immersed in 0.5% PAS (Sigma–Aldrich, St. Louis, MO) for 10 min, rinsed in dH_2O, and exposed to Schiff's reagent (Electron Microscopy Sciences, Hatfield, PA). Subsequently, the slides were rinsed in dH_2O and immersed in Harris hematoxylin (Sigma Aldrich) for 20 s. After rinsing in dH_2O, the slides were subjected to dehydration through an ethanol gradient and clearing in xylene and then were mounted in Permount medium (Fisher Scientific, Pittsburgh, PA) and digital images were captured from at least 10 random fields. Acellular capillaries and pericyte loss were identified based on prominent histological characteristics.

All data are reported as mean ± SD; one-way ANOVA followed by a Student t test was used to analyze all data. In addition, the relationship between the number of acellular capillaries or pericyte loss and Cx43 expression was analyzed by Pearson correlation

Table 1
Medical history of eye donors.

	Age	Sex	Duration of DR (yrs)	#AC	#PL	Post-mortem time	Cause of death
Non-diabetic	89	F	0	2	3	4:45	Probable pneumonia
	90	F	0	1	3	6:35	Cerebrovascular accident
	61	M	0	2	2	6:00	Accident
	90	M	0	3	3	8:50	Unknown
	86	F	0	3	4	6:05	Cardiac/heart disease
	85	F	0	4	6	6:56	Unknown
Diabetic	80	F	1	5	6	9:00	Unknown
	88	M	4	9	7	2:33	Unknown
	64	M	4	8	9	8:10	Anoxic encephalopathy
	93	F	8	12	14	6:20	Intracranial hemorrhage
	77	F	9	4	6	8:00	Pneumonia
	78	M	9	7	11	6:12	Heart failure

#AC and #PL refer to the number of acellular capillaries or pericyte loss per ~16 mm^2 of retina, respectively.