Tight Glycemic Control Regulates Fibronectin Expression and Basement Membrane Thickening in Retinal and Glomerular Capillaries of Diabetic Rats

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PURPOSE. To determine whether tight glycemic control prevents development of basement membrane (BM) thickening in retinal and glomerular capillaries of diabetic rats and whether the extent to which BM thickening develops is linked to fibronectin (FN) overexpression and the degree of hyperglycemia.

METHODS. Retinal and renal cortical tissues obtained from the tightly controlled diabetic (TC), poorly controlled diabetic (D), and nondiabetic (N) control rats were subjected to morphometric and biochemical analyses. In both tissues, capillary BM thickening was determined by electron microscopy, and FN protein level was assessed by Western blot analysis. Routine measurements of blood glucose level and glycohemoglobin level were performed throughout the study.

RESULTS. The Hba1c level was significantly increased in D rats, but not in TC rats, compared with those of the N rats with a concomitant increase in capillary BM thickness and FN protein expression in retinial and renal tissues. A strong correlation was observed between retinal and glomerular capillary BM thickness (r = 0.79, P = 0.0001), between retinal and kidney FN protein levels (r = 0.7, P = 0.005), between Hba1c and FN protein levels in the retina (r = 0.66, P = 0.006) and kidney (r = 0.84, P = 0.0003), and between Hba1c level and BM thickness in retinal (r = 0.76, P = 0.0002) and renal tissues (r = 0.64, P = 0.004).

CONCLUSIONS. In diabetes BM thickening develops in retinal and glomerular capillaries in a correlated manner. Tight glycemic control may be beneficial in preventing the pathologic development of capillary BM thickening and FN overexpression in retinal and renal tissues, two target tissues of diabetic microangiopathy. (Invest Ophthalmol Vis Sci. 2009;50: 943–949) DOI:10.1167/iovs.08-2377

Vascular BM thickening is the most characteristic structural abnormality of small blood vessels in diabetic retinopathy and diabetic nephropathy.1–16 Results from the Diabetes Control and Complication Trial, the UK Prospective Diabetes Study, and other studies have clearly established hyperglycemia as a causative factor underlying the development of microvascular complications.7–9 Numerous studies have established that hyperglycemia plays a significant role in the development of vascular BM thickening in retinal and glomerular capillaries. We have shown that a high-glucose condition, or hyperglycemia, upregulates the expression of BM components, FN, collagen type IV, and laminin10,11 and contributes to the development of vascular BM thickening and increased retinal vascular permeability.12 Although independent studies have investigated the development of BM thickening in either the retinal13–16 or the glomerular capillaries17–19 in diabetes, none has examined the effect of tight glycemic control in both retinal and glomerular vessels simultaneously with respect to BM component overexpression and thickening. Therefore, it remains unclear whether capillary BM thickening in diabetes develops in a correlated manner in retinas and kidneys, two of the most important target tissues of diabetic microangiopathy.

Although thickening of capillary BMs is considered an index of the progression of microvascular complication in diabetes, scant information exists in the literature regarding its rate of development in multiple tissues. A recent study examining BM thickening in retinal and glomerular capillaries of OVE26 transgenic diabetic mice reported a greater increase in glomerular BM thickening than in retinal BM thickening.21 However, in this study, it was unclear whether the progression of BM thickening developed in a correlated manner in the retinal and glomerular capillaries. Although some studies suggest an association between the development of vascular changes in diabetic retinopathy and those seen in diabetic nephropathy,22 others have noted marked discordance between development of diabetic retinopathy and nephropathy, despite significant correlation between severity of diabetic retinopathy and increased glomerular BM thickness.23 Our recent studies suggest that hyperglycemia-induced overexpression of BM components not only contributes to retinal capillary BM thickening but also to increased retinal vascular permeability. Overall, these studies indicate that our understanding of the role these structural changes play in diabetic microangiopathy is incomplete. Although we have advanced our understanding and have established the importance of hyperglycemia-induced overexpression of BM components24–26 in the development of capillary BM thickening,25,26,29–32 the relative effect of hyperglycemia on vascular BM thickening in different tissues remains unclear.

A major reason for studying vascular BM thickening in diabetic microvascular complications is to understand how concordance or discordance in the development of structural changes could affect the development of other lesions of diabetic retinopathy and diabetic nephropathy. Therefore, the purpose of this study was to determine whether the development of BM thickening in the retinal and glomerular capillaries in diabetes occurs in a correlated manner, the extent to which the development of BM thickening is linked to FN overexpression and level of hyperglycemia, and whether tight glycemic control prevents BM thickening simultaneously in the two target tissues.

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Supported by National Eye Institute Grant EY14702, the American Diabetes Association, and in part by a departmental grant from the Massachusetts Lions Eye Research Fund, Inc.

Submitted for publication June 4, 2008; revised August 20, 2008; accepted November 21, 2008.

Disclosure: S. Cherian, None; S. Roy, None; A. Pinheiro, None; S. Roy, None.

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MATERIALS AND METHODS

Streptozotocin-Induced Diabetes

Diabetes was induced in 16 Sprague-Dawley rats with streptozotocin injection at 55 mg/kg body weight. Hyperglycemia in these rats was verified by measuring blood glucose levels 2 or 3 days after streptozotocin injection. Diabetic rats with moderate blood glucose levels between 125 and 250 mg/dl (n = 9) were selected as the poorly controlled diabetic (D) group, and the nondiabetic (N) rats (n = 7) represented the N group. Insulin (NPH) was administered to all diabetic rats to allow a steady gain in body weight without ketosis. The poorly controlled diabetic rats received 2 to 4 units of insulin on alternate days, and the tightly controlled diabetic rats received insulin twice daily (3–5 units per injection). The blood glucose level was measured biweekly with a glucometer (OneTouch Ultra; Lifescan, Inc., Milpitas, CA), and the insulin dose was adjusted as needed. The HbA1c level was measured in all animals with hand-held meters (A1cNow; Bayer Corp., Robinson Township, PA) at 3-month interval. All rats entered in the study were also monitored weekly for their body weight and blood glucose level during the study and were given free access to water and food and maintained under a 12 hour-on/12 hour-off light cycle. After 6 months of diabetes, the rats were killed, and the retinal and kidney cortical tissues were processed for biochemical and morphometric analyses. To establish baseline values for retinal and glomerular capillary BM thickness four rats were killed at the start of the study. All experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Electron Microscopy

Retinas and kidney cortex tissues were immediately fixed after isolation in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. The tissues were dehydrated using osmium tetroxide, ethanol, and propylene oxide (EMS, Hatfield, PA). The tissues were then embedded in an Epon-Araldite plastic mixture and baked for 48 hours. Ultrathin sections were serially cut at 60 to 70 nm with a microtome (LKB Ultratome; Nova, Bromma, Sweden). The sections were collected, placed on a copper grid, stained with 4% uranyl acetate (EMS) in methanol, and viewed by transmission electron microscope (Philips Electron Optics, Eindhoven, Netherlands). At least 10 random images of retinal capillaries from the outer plexiform and ganglion cell layers and glomerular capillaries from each animal were obtained and enlarged to 50,000× magnification for examination. These electron micrographs were analyzed according to the orthogonal intercept method33 for BM thickness.

Measurement of Retinal Capillary BM Width and Retinal Histopathology

Retinal capillary BM width was determined from electron micrographs using the method of Siperstein et al.33 In brief, using a computer with image-management software (Photoshop, Adobe Systems, Mountain View, CA), a 20-spoke radial grid was superimposed over each capillary micrograph, and the thickness of the BM was measured at each point a where a spoke intersected the BM. The width of the two thinnest portions of the BM surrounding each capillary was also measured. Most measurements of BM width were performed on capillaries from the outer plexiform layer of the mid retina. At least 10 capillaries were photographed and measured from each of the 10 retinal sections per animal to determine capillary BM thickness. Overlapped areas from BM of pericytes and endothelial cells were excluded from analysis. Data were collected and evaluations performed in a masked manner as regarded the treatment group of each rat.

Western Blot Analysis

Western blot analysis was performed to determine the relative level of FN protein in the rat retina and kidney, according to the method of Towbin et al.34 Rat retinas and kidneys were dissected and placed in buffer containing 25 mM Tris (pH = 7.4), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100. These samples were then homogenized and centrifuged, and the supernatant used to determine the protein levels. Total protein was determined by BCA protein assay (Pierce Chemical, Rockford, IL), and volumes representing equal amounts of total protein were electrophoresed on a 6% SDS-PAGE with molecular weight markers (Bio-Rad, Hercules, CA). After electrophoresis, the gel was transferred onto a nitrocellulose membrane. The membrane was blocked for 2 hours with 5% nonfat dry milk solution in TTBS. The membrane was then washed and incubated with rabbit anti-FN antibody (1:1,000; Chemicon, Temecula, CA) followed by anti-rabbit IgG (1:15,000; Sigma-Aldrich, St. Louis, MO). The membrane was washed again and allowed to react with chemiluminescent enzyme substrate (Bio-Rad). It was then exposed to film (Fuji, New York, NY) and densitometric analysis performed with NIH Image software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

Statistical Analysis

All data are expressed as the mean ± SD. Statistical analysis was performed by ANOVA and Student’s t-test. The correlation between retinal and glomerular BM thickening, between FN protein levels and BM thickening, and between HbA1c and BM thickening was analyzed by Pearson correlation coefficient, P < 0.05 was considered significant.

RESULTS

Routine measurements of blood glucose level and periodic glycohemoglobin level confirmed the status of the three groups: N, TC, and D. We present evidence that capillary BM thickening is closely linked to the level of hyperglycemia and FN protein expression in retinal and renal tissues of diabetic rats. In addition, we observed that development of BM thickening and levels of hyperglycemia correlated, as did BM thickening and FN protein levels. These observations indicate that tight glycemic control may be effective in reducing FN overexpression and capillary BM thickening.

Correlation between BM Thickness in Retinal and Glomerular Capillaries of Diabetic Rats

BM thickening in retinal and glomerular capillaries developed in a correlated manner during 6 months of streptozotocin-induced diabetes (r = 0.79; P = 0.0001; Figs. 1A, 1B). The mean BM thickness of retinal and glomerular capillaries was significantly increased in the D rats compared with those of N rats (69.2 nm vs. 50.8 nm, P < 0.02; 111.4 nm vs. 81 nm, P < 0.02, respectively), a 56% and 38% increase, respectively. The BM thickness of retinal and glomerular capillaries of TC rats was slightly increased (5.5% and 7.0%, respectively) but not statistically significant, when compared with those of N rats (53.6 nm vs. 50.8 nm, P = 0.28; 86.7 nm vs. 81 nm, P = 0.25; Fig. 1C). The data on retinal and glomerular capillary BM thickness of each animal is presented in Figure 1B. Although the percentage increase in BM thickness during the 6 months of diabetes was similar for retinal and glomerular capillaries in the D rats (43% vs. 40.5%, adjusted for aging, respectively), the actual capillary BM thickening in the two tissues was different; the retinal capillaries thickened by an average of 26.2 nm whereas the glomerular capillaries thickened by 41.4 nm. The BM thickness of glomerular capillaries was consistently greater than those of the retinal capillaries. On the basis of the baseline values obtained at the start of the study for retinal and glomerular capillary BM thickness (43 ± 7 nm, 74 ± 5 nm, respectively) compared with the BM thickness after 6 months (50.8 and 8 nm, respectively), aging and other nondiabetic factors appear...
to have contributed to overall BM thickening by approximately 18% and 10% in these two tissues, respectively.

**Correlation between HbA1c Level and BM Thickness in Retinal and Glomerular Capillaries**

HbA1c levels of D rats compared with those of N or TC rats were significantly increased (11.0% ± 2.6% vs. 5.6% ± 0.7%, \(P = 0.0007\); 11.0% ± 2.6% vs. 7.8% ± 0.3%, \(P = 0.01\), respectively; Fig. 2). A strong correlation was observed between HbA1c level and BM thickness of retinal capillaries (\(r = 0.76, P = 0.0002\)), and between HbA1c and glomerular capillaries (\(r = 0.64, P = 0.004\); Figs. 3A, 3B). Of note, the D rat with the highest HbA1c level exhibited the highest BM thickness in both the retinal and glomerular capillaries, and the nondiabetic control rat with the lowest HbA1c level exhibited the lowest BM width for the retinal capillaries and the second lowest BM width for the glomerular capillaries.

**Correlation between Diabetes-Induced Upregulation of Fibronectin Protein Level in Retinal and Renal Tissues of Diabetic Rats**

Western Blot analysis indicated that FN protein expression was significantly increased in the retinas of poorly controlled diabetic (D) rats compared with those of nondiabetic control (N) or tightly controlled diabetic (TC) rats (188.7% ± 26% vs. 92.4% ± 32.5%, \(P = 0.001\); 188.7% ± 26% vs. 105.3% ± 22%, \(P = 0.002\); Figs. 4A, 4B, 4C). Similarly, Western Blot analysis indicated that FN protein expression was significantly increased in the renal cortex of D rats compared with those of N or TC rats (240.7% ± 69% vs. 101.6% ± 22%, \(P = 0.002\); 240.7% ± 69% vs. 117.6% ± 12.5%, \(P = 0.002\), respectively). Tight glycemic control prevented FN overexpression in the retinal or renal tissues of TC rats and showed no statistically significant difference compared with that in the N rats (Figs. 4A, 4B, 4C). When FN protein levels in the retinal and renal tissues were compared in the diabetic rats, a statistically significant correlation was observed (\(r = 0.7, P = 0.005\); Fig. 4C).

**Correlation between FN Protein Level and BM Thickening in Retinal and Glomerular Capillaries of Diabetic Rats**

A strong correlation was observed between capillary BM thickening and the level of FN protein expression in retinas of diabetic rats (\(r = 0.91, P = 0.0001\); Fig. 5A). Similarly, BM thickening in glomerular capillaries and FN expression in kidney cortex of diabetic rats showed significant correlation (\(r =
0.63, \( P = 0.007 \); Fig. 5B). Of interest, the two rats with the highest FN protein expression also exhibited maximum BM thickening in both their retinal and glomerular capillaries.

**Correlation between HbA1c or Blood Glucose Levels and FN Protein Level in Retinas and Kidney Cortex of Diabetic Rats**

HbA1c level in the D, TC, and N rats showed strong correlation with their corresponding FN protein level in the retinas (\( r = 0.66, P = 0.006 \); Fig. 6A). Similarly, the HbA1c level in these rats showed a strong correlation compared with the corresponding FN protein level in the kidney cortex (\( r = 0.84, P = 0.00003 \); Fig. 6B). Initial and final blood glucose levels and corresponding retinal and glomerular capillary basement membrane thickness of control, tightly controlled, and poorly controlled diabetic rats are presented in Table 1. The blood glucose concentrations showed a significant correlation with BM thickening of retinal (\( r = 0.67, P = 0.003 \)) and glomerular (\( r = 0.62, P = 0.006 \)) capillaries.

**DISCUSSION**

To evaluate the extent to which vascular BM thickening is affected by hyperglycemia in retinal and glomerular tissues in diabetes and whether institution of tight glycemic control has a bearing on its development, we assessed capillary BM thickening in the retinal and renal cortical tissues of rats with 6 months of diabetes under tight glycemic control and compared them to those in poorly controlled diabetic rats. After 6 months

**FIGURE 3.** (A) Correlation between HbA1c level and corresponding BM thickness in retinal capillaries of N, TC, and D rats. Each dot represents an individual experimental animal, and the line connecting paired symbols shows the relationship between HbA1c levels and retinal capillary BM thickness. (B) Graphic illustration showing correlation between HbA1c level and corresponding BM thickness in glomerular capillaries of N, TC, and D rats. Symbols are as described in (A).

**FIGURE 4.** (A) Representative Western blots showing the FN protein level in retinal and kidney cortical tissues in rats of the three groups: N, TC, and D. Note the retinal and kidney protein was obtained from the same animal. The FN protein level was significantly increased in the retinal and renal tissues of D rats compared with those of the N or TC rats. Bottom: images of corresponding Ponceau-S-stained membranes. (B) Percentage of FN protein level in the N, TC, and D groups. Retinal FN expression was significantly increased in the poorly controlled diabetic group compared with those of either the N or TC groups (left). Glomerular FN expression was significantly increased in the poorly controlled diabetic group compared with those of either N or TC groups (right). (C) Correlation between retinal and kidney cortical FN levels in N, TC, and D rats. Each symbol represents an individual experimental animal, and the line connecting paired symbols shows the relationship between FN protein levels of retinal and glomerular capillaries of each animal.
of diabetes, BM thickening developed in all diabetic rats. However, the extent of BM thickening varied from animal to animal in both the retinal and glomerular capillaries. Comparison of retinal and renal capillary BM thickness revealed that the development of BM thickening occurred in a correlated manner ($r = 0.79$) in the two tissues of each animal. In the TC rats, tight glycemic control inhibited capillary BM thickness, which was essentially similar to those of the N rats, indicating a beneficial effect of good glycemic control in preventing BM thickening. We also observed a strong correlation between capillary BM thickness and the HbA1c or the FN protein levels.

Although vascular BM thickening is closely associated with the development of diabetic retinopathy and diabetic nephropathy, it is unclear the extent to which development of BM thickening must occur to trigger capillary dysfunction. Variability in capillary BM thickness has been shown to be linked to severity in functional complications. For example, a previous study has demonstrated that the severity of diabetic retinopathy correlates with increased glomerular BM width derived from renal biopsies. Similarly, another study reported that muscle capillary BM thickening of patients with proliferative retinopathy was significantly greater than that observed in the patients without retinopathy. Although various factors including individual genetics are likely to play a role in regulating BM thickness that may contribute to subsequent organ dysfunction, it is evident that the development of diabetic microvascular complications is, at least in part, related to capillary BM thickness.

Thickened BMs may compromise tissue function by several mechanisms. Studies examining anionic site distribution in glomerular BMs reported reduced charge density and impaired exchange of metabolites due to thickening of the glomerular BM in patients with diabetic nephropathy. Abnormal vascular BM thickening in diabetes leads to interference in cell-matrix interaction, alters pore size in glomerular BM, and induces pericyte loss and acellular capillaries in diabetic retinas. Taken together, capillary BM integrity is a critical factor in the regulation of cellular functions in target tissues of diabetes.

Overexpression of BM components has been well documented in retinal and renal tissues in diabetes, and the inhibition of this abnormality has been shown to prevent BM thickening with a beneficial effect on vascular permeability.

**Figure 5.** (A) Correlation between retinal capillary BM thickness and retinal FN protein levels in N, TC, and D rats. Each symbol represents an individual experimental animal, and the line connecting the paired symbols shows the relationship between retinal capillary BM thickness and FN protein levels. (B) Graphic illustration showing the correlation between glomerular capillary BM thickness and renal FN protein levels in N, TC, and D rats. Symbols are as described in (A).

**Figure 6.** (A) Correlation between HbA1c levels and retinal FN protein expression in N, TC, and D rats. Each symbol represents an individual experimental animal, and the line connecting the paired symbols shows the relationship between HbA1c and retinal capillary FN protein levels. (B) Correlation between HbA1c levels and kidney FN protein expression in N, TC, and D rats. Symbols are as described in (A).
However, the effect of tight glycemic control on FN overexpression and development of BM thickening simultaneously in both the retinal and renal tissues in diabetes has not been carefully examined. In this study, FN overexpression and the level of hyperglycemia influenced the development of capillary BM thickening in diabetic retinal and glomerular tissues. The findings suggest that one of the mechanisms by which tight glycemic control prevents BM thickening is by downregulating FN overexpression and the development of capillary BM thickening and subsequent organ dysfunction. Further studies could help determine the beneficial effects of reduced BM thickening in regulation of functional lesions associated with diabetic retinopathy and diabetic nephropathy.

References


Blood glucose (BG) concentrations showed a significant correlation with BM thickening of retinal and glomerular capillaries.

Table 1. Blood Glucose Levels and Corresponding BM Thickness in Retinal and Glomerular Capillaries of N, TC, and D Rats

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<th>Final BG (mg/dL)</th>
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<th>Kidney BM Thickness</th>
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Initial (nm) | Final (nm) | Initial (nm) | Final (nm) |
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