Downregulation of Fibronectin Overexpression Reduces Basement Membrane Thickening and Vascular Lesions in Retinas of Galactose-Fed Rats

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Overexpression of extracellular matrix (ECM) components is closely associated with the development of vascular basement membrane (BM) thickening, a histological hallmark of diabetic microangiopathy. To determine whether BM thickening of retinal capillaries could be prevented by down regulating synthesis of fibronectin, an ECM component, we used antisense oligos targeted against translation initiation site of the fibronectin transcript in galactose-fed rat, an animal model of diabetic retinopathy. After 2 months of galactose-feeding, intravitreal administration of 3 μmol/l antisense fibronectin oligos was initiated at monthly intervals for 3 months. The antisense strategy significantly reduced fibronectin mRNA and protein level in the retinas of treated eyes compared with untreated eyes of galactose-fed rats (130 ± 16 vs. 179 ± 18% of control, $P < 0.01$, and 144 ± 28 vs. 204 ± 22% of control, respectively, $r = 0.9$) and resulted in partial reduction of retinal capillary BM width (123 ± 16 vs. 201 ± 12 nm, $P < 0.03$). In eyes treated with antisense fibronectin oligos, ~35% reduction in both pericyte loss and acellular retinal capillaries was observed ($P < 0.04$ and $P < 0.03$, respectively). Glycohemoglobin level was consistently elevated in the treated (6.9 ± 0.6%) and untreated (6.5 ± 0.7%) galactose-fed rats compared with control rats (4.5 ± 0.8%). Overall, these results indicate that downregulation of fibronectin synthesis reduces BM thickening in retinal capillaries with beneficial effect to retinal lesions. The antisense fibronectin oligos may provide a useful approach for reducing vascular lesions in diabetic retinopathy. The thickened vascular BM may be a potential therapeutic target for preventing retinal lesions in diabetic retinopathy. *Diabetes* 52: 1229–1234, 2003

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Received for publication 9 September 2002 and accepted in revised form 14 February 2003.

BM, basement membrane; ECM, extracellular matrix; ILM, inner limiting membrane.

Vascular basement membrane (BM) thickening is a fundamental structural abnormality of diabetic microangiopathy (1–5). In diabetes, vascular BM thickening is a slowly developing process when extracellular matrix (ECM) accumulation occurs. While numerous studies suggest that this structural lesion develops, at least in part, from excess synthesis and accumulation of BM material in the vessel walls, none have proven that excess synthesis of the BM components actually contributes to the development of the thickened vascular BMs in diabetic microangiopathy (6–14).

Several studies including ours have shown that fibronectin synthesis is upregulated by high glucose or diabetes (10,11,13,15–17). Fibronectin, a principal adhesive protein with several distinct binding sites for various ECM components, crosslinks and forms the BM core structure. Fibronectin also facilitates cell attachment via integrins to the BM and plays an important role in cell proliferation and migration during wound healing in diabetes (18–20). Thus, altered fibronectin synthesis may have serious pathological consequences for development of structural lesions of diabetic retinopathy.

To determine whether fibronectin gene upregulation plays a role in the development of vascular BM thickening, a strategy using antisense technology was developed to reduce fibronectin gene expression (21). In previous studies we have documented that intravitreal administration of fibronectin antisense oligos reduced fibronectin synthesis in the retinal vascular cells (22). With this strategy in place, we determined whether reduction of fibronectin overexpression would prevent the development of vascular BM thickening in diabetic retinopathy. We assessed whether fibronectin upregulation played a role in the development of BM thickening in galactose-fed rats, an animal model of diabetic retinopathy (23) that develops retinal vascular lesions consistent with the early stages of diabetic retinopathy, including BM thickening.

The use of antisense oligos to inhibit specific gene expression is a powerful approach for analysis of gene function with the potential for application in gene therapy. In this study, we have used the antisense approach to test its potential applicability in vivo toward preventing the development of BM thickening and to determine whether reducing BM thickening may prevent the development of retinal lesions in diabetic retinopathy. Our recent finding that fibronectin synthesis can be reduced in the rat retina

with antisense oligos targeted to the fibronectin transcript provided a valuable tool toward downregulation of fibronectin expression in vivo.

We have previously documented that retinal fibronectin expression is upregulated early in galactose-fed rats, which provided background information regarding the initiation of fibronectin oligo treatment (11). In this study, we have determined whether reduction of fibronectin overexpression in the galactose-fed rat retina prevented the development of BM thickening in retinal capillaries and blocked the development of characteristic vascular lesions of diabetic retinopathy.

RESEARCH DESIGN AND METHODS

Sprague-Dawley male rats weighing ~200 g were randomly assigned to four groups: rats fed regular diet, galactose-fed rats, galactose-fed rats treated with antisense oligos, or galactose-fed rats treated with random oligos. After 2 months of 30% galactose diet, rats representing the antisense oligo-treated group were intravitreally injected via pars plana, using a 33-gauge beveled needle under a dissecting microscope. The intravitreal injections were performed with 3 μmol/l fibronectin antisense oligos at monthly intervals for 3 months. Similarly, rats representing the random oligo-treated group were intravitreally injected with 3 μmol/l random oligos at monthly intervals for 3 months. After 2 months postintravitreal injection, that is, after 7 months from initiation of regular or galactose diet, rats from the four groups were killed. Glycohemoglobin levels were measured in each animal at ~3-month intervals using a commercially available kit (Glyc-Affin; Pierce, Rockford, IL). Consistent with our previous finding (22), no toxic effect from the delivery of the antisense oligos was observed. All experiments conformed to the Association for Research in Vision and Ophthalmology (ARVO) Resolution on the Use of Animals in Research.

Antisense oligonucleotide treatment. A 17-mer antisense oligonucleotide (5′-CCTGAGCATCTTGAGTG-3′) encompassing the translation initiation site of the fibronectin transcript was used to reduce fibronectin expression in rat retinal vascular cells. The 17-mer oligos sequence, which was phosphorothioate modified, was previously shown to be effective in reducing fibronectin expression in cultured rat endothelial cells and in rat retinas (21,22). The oligo-polymer complexes were produced by mixing 3 μmol/l oligos with 6 μmol/l POE-PSP and utilized within an hour after the preparation.

RT-PCR. RT-PCR was performed to determine relative level of fibronectin mRNA in the retina of galactose-fed rat eyes, antisense oligo-injected eyes, random sequence oligo-injected eyes, and control eyes. The retinas were dissected under sterile RNase-free conditions, and total RNA was isolated using the Total RNA Isolation kit (Promega, Madison, WI). Reverse transcription was processed in parallel with the retinal RNA from all four groups. An endogenous (β-actin) mRNA was used as an internal control for assessing relative level of fibronectin mRNA as previously described (9). To prevent quantitative inaccuracies, separate tubes containing increasing volumes (2 and 4 μl) of the RT reaction were amplified. The fibronectin and the actin primers bridged introns; this feature allowed detection of any contaminating genomic DNA. The fibronectin primer pair encompassed the alternatively spliced EIIIA exon (270 bp) and generated two RT-PCR products: the 852-bp fragment that included the exon and the 582-bp fragment that excluded the exon. The sequences of the rat fibronectin and actin primer pair have been previously reported (21). The PCR cycle conditions were the same for fibronectin and β-actin cDNA, except for the number of cycles: denaturation for 1 min at 95°C, annealing for 1 min at 54°C, and extension for 2 min at 72°C with 5 min extension at the end of the reaction for 30 and 28 cycles, respectively. PCR products from retinal RNA representing the four groups were always resolved on the same gel. The densitometric values of RT-PCR products generated with increasing volumes of cDNA were averaged to yield the fibronectin and actin signals for each sample (densitometric units/microliter RT reaction).

Western blot analysis. Western blot analysis was performed to determine relative level of fibronectin protein in rat retinas as described previously (22). Briefly, rat retinas were dissected out and placed in buffer containing 25 mmol/l Tris, pH = 7.4, containing 1 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride, and 0.1% Triton X-100. The samples were homogenized and the supernatant used in subsequent steps. Total protein was determined by BCA protein assay (Pierce Chemical, Rockford, IL), and volumes representing equal amount of protein were used for immunoprecipitation. The retinal fibronectin protein from each sample was exhaustively immune precipitated from the total protein with rabbit anti-rat fibronectin, diluted 1:500, (Chemicon, Temecula, CA). The resulting immune complexes were adsorbed with protein-G agarose (CalBiochem, La Jolla, CA), resuspended in Laemmli buffer containing β-mercaptoethanol and electrophoresed on a 10% SDS-PAGE, together with molecular weight markers (BioRad, Hercules, CA). Following electrophoresis, the gel was transferred as previously described (21). The membrane was prewetted, blocked for 90 min with 5% BSA in Tris-buffered saline and replaced with fresh blocking solution containing rabbit anti-rat fibronectin antiserum (GIBCO) diluted at 1:1,000 for 3 h. The membrane was washed and incubated with a goat anti-rabbit antibody conjugated with horseradish peroxidase (diluted 1:5,000) for 30 min. The membrane was washed as above, equilibrated in appropriate buffer for 5 min, and then allowed to react to the enzyme substrate (Kodak). The membrane was subsequently exposed to film. And densitometric analysis was performed at nonsaturating exposures with a laser scanning densitometer.

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. (24). In brief, a 20-spoke grid was applied over each capillary micrograph, and thickness of the BM at each point that a spoke intersected the BM was measured. 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 midretina. Approximately 10 capillaries were photographed and measured from retinal sections per animal to determine capillary BM thickness. Data were collected and histopathological evaluations performed in a masked manner to keep the identity of the treatment unknown.

Retinal trypsin digest preparation. To analyze the retinal vasculature for pericyte loss and acellular retinal capillaries, we used retinal trypsin digest technique as described by Kuwabara and Cogan (25) with minor modifications. Briefly, after enucleation eyes were fixed in 10% formalin, intact retinas were dissected out and subjected to 3% trypsin digestion at 37°C for 2–3 h with gentle shaking. Under the dissecting microscope the nonvascular mass was removed from the entire vascular network then mounted and stained with Periodic Acid Schiff and hematoxylin. The slides were immersed in 0.5% periodic acid (Sigma) for 10 min, rinsed in water, and reacted in Schiff's reagent (Sigma) for 10 min. After a water rinse the slides were dipped in acid ethanol (1% HCl), rinsed, and reacted in 1% $LiCO_3$. After dehydration in ethanol and clearing in xylene, the slides were mounted with Permount. The PAS stained the glycoprotein of the BMs in pink, while the cellular nuclei (elliptical and oriented along the circumference of the capillary cross-section for endothelial cells and round, abutting the outer portion of the capillary wall for pericytes) were stained blue with the hematoxylin. Representative areas of the vascular network was photographed using a Kodak digital camera, and the images were analyzed for retinal morphology, in particular, pericyte loss, and acellular capillaries.

Estimation of pericyte loss and acellular capillaries. Approximately 1,200 capillary cells were counted from the midretinal area of each retina following trypsin digest and hematoxylin and eosin staining. At first, the images of retinal capillaries were captured using a Kodak digital camera connected to a computer and then pericyte counts determined from the images. Specific loss of pericyte could be detected from the empty "shell" left behind after pericyte "dropout." Capillaries that lacked both pericytes and endothelial cells were considered acellular. A defined area (1 mm^2) was analyzed for each of the treated and the untreated groups to compare the number of acellular capillaries.

Statistical analysis. The data are reported as means ± SD; one-way ANOVA followed by a Student-Newman-Keuls test was used to analyze all data. Data with values of $P < 0.05$ were considered significant.

RESULTS

In all rats entered in this study, periodic glycohemoglobin measurements and routine eye examinations were performed. Glycohemoglobin levels, monitored at 3-month intervals were consistently elevated in the galactose-fed rats compared with control rats (6.9 ± 0.6 vs. 4.5 ± 0.8%, $P < 0.01$). At the time of death, the glycohemoglobin level measurement confirmed the presence of hyperhexosemia in the galactose-fed rats compared with control rats (7.3 ± 1.1 vs. 4.8 ± 1%, $P < 0.002$). Consistent with our previous report, eye examinations performed in uninjected eyes and intravitreally injected eyes revealed no sign of redness, inflammation, or "milky/cloudy" appearance.

Downregulation of fibronectin mRNA level in rat retina treated with antisense fibronectin oligonucle-

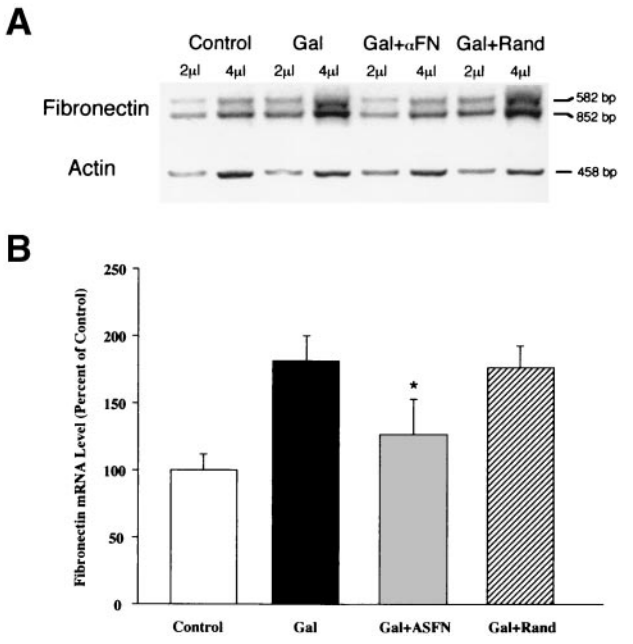

FIG. 1. Relative levels of fibronectin mRNA in rat retinas. *A*: RT-PCR amplification products for fibronectin and β-actin after electrophoresis on GelStar-stained agarose gel using 2 and 4 μl of cDNA from retinas of control rat eyes, galactose-fed rat eyes, galactose-fed rat eyes injected with antisense fibronectin (ASFN) oligos, or random (Rand) sequence oligos. Fibronectin PCR primer pair encompassing alternatively spliced EIIIA exon produces two bands: 582 bp and 852 bp; the actin primer pair generates a single 458-bp band. *B*: Graphical illustration of fibronectin mRNA level showing selective reduction of fibronectin mRNA level in retinas of galactose-fed rat eyes injected with antisense fibronectin oligos. β-Actin mRNA level is not altered by high galactose concentration. Fibronectin mRNA level is shown as percent of control with respect to retinal fibronectin mRNA from normal rat (n = 5 for each group).

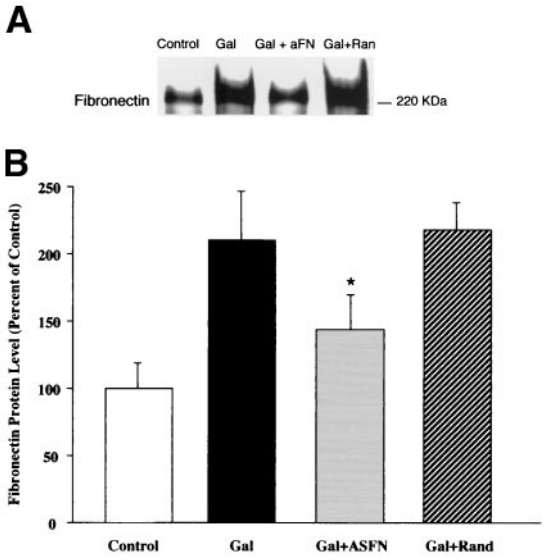

FIG. 2. *A*: Western blot analysis of relative amount of fibronectin protein in rat retinas from control rat eyes, galactose-fed rat eyes, and galactose-fed rat eyes injected with antisense fibronectin (ASFN) oligos or random (Rand) sequence oligos. *B*: A graphical illustration showing fibronectin protein level is reduced in galactose-fed rat eyes injected with antisense fibronectin oligos compared with that of galactose-fed rat eyes (n = 5 for each group).

otide. RT-PCR analysis indicated that after 7 months of galactose-feeding, the retinal fibronectin mRNA level was significantly increased in the galactosemic rats compared with the control rats fed on regular diet (101 ± 14 vs. 179 ± 21% of control, $P < 0.0001$, $n = 5$) (Fig. 1*A* and *B*). In galactosemic rat eyes that received antisense fibronectin oligo, fibronectin mRNA level was consistently decreased compared with the galactosemic rats (130 ± 25 vs. 179 ± 21% of control, $P < 0.03$, $n = 5$) (Fig. 1*A* and *B*). The amount of β-actin mRNA level, used as internal control, remained unaltered in treated and control eyes. Also, the fibronectin level in the random oligo-treated eye showed no change. Together, the data indicated specificity of the fibronectin antisense oligos in reducing fibronectin mRNA level in the rat retinas.

Downregulation of fibronectin protein level in rat retina treated with antisense fibronectin oligonucleotide. Western blot analysis indicated that after 7 months of galactose-feeding, the retinal fibronectin protein level was significantly increased in the galactosemic rats compared with the control rats fed on regular diet (100 ± 22 vs. 204 ± 37% of control, $P < 0.003$, $n = 5$) (Fig. 2*A* and *B*). Western blot analysis indicated that the steady-state level of fibronectin protein was significantly reduced (144 ± 28 vs. 204 ± 22% of control, $P < 0.04$, $n = 5$) in retinas of galactosemic rat eyes injected with fibronectin antisense oligos (Fig. 2*A* and *B*). The eyes injected with random oligos showed no change in the fibronectin protein level compared with that of the galactosemic group. The reduction in fibronectin protein level correlated with the relative reduction in fibronectin mRNA level ($r = 0.9$).

Reduced vascular BM thickening in rat retina injected with antisense fibronectin oligonucleotides. Retinal capillary BM width measured in uninjected control eyes compared with eyes from galactosemic rats showed a significant difference; consistent with previous reports, the retinal capillary width was significantly increased in the galactosemic rats (85 ± 20 vs. 139 ± 16 nm, $P < 0.002$, $n = 5$) (Fig. 3*A* and *B*). The BM width in retinal capillaries of galactosemic rat eyes injected with antisense oligos showed a significant reduction compared with those of the galactosemic rat eyes (105 ± 24 vs. 139 ± 16 nm, $P < 0.03$, $n = 5$) (Fig. 3*B* and *C*). Galactosemic rat eyes injected with random oligos showed no change in the retinal capillary BM width compared with the retinal capillaries of uninjected galactosemic rat eyes (Fig. 3*B* and *D*). Although the fibronectin antisense oligos significantly reduced BM thickening, the oligos did not completely reduce BM thickening to the level of the control rats.

Decreased number of pericyte loss and acellular capillaries in retinas with reduced vascular BM thickening. The two histopathological lesions, pericyte loss and acellular capillaries assessed in this study showed significant improvement in eyes treated with fibronectin antisense oligos. The number of pericyte loss and acellular vessels that was significantly increased in retinas of galactosemic rats compared with control rats (2.4-fold of control, $P < 0.001$, and 2.2-fold of control, $P < 0.004$, $n = 6$, respectively), showed a significant reduction in the galactosemic eyes treated with fibronectin antisense oligos (2.4- vs. 1.6-fold of control, $P < 0.04$, and 2.2- vs. 1.4-fold of control, $P < 0.03$, $n = 6$, respectively) compared with untreated galactosemic rats (Fig. 4*A* and *B*). The number of pericyte loss and acellular vessels of galactosemic rats that received random oligos were not different from those of the galactosemic rats.

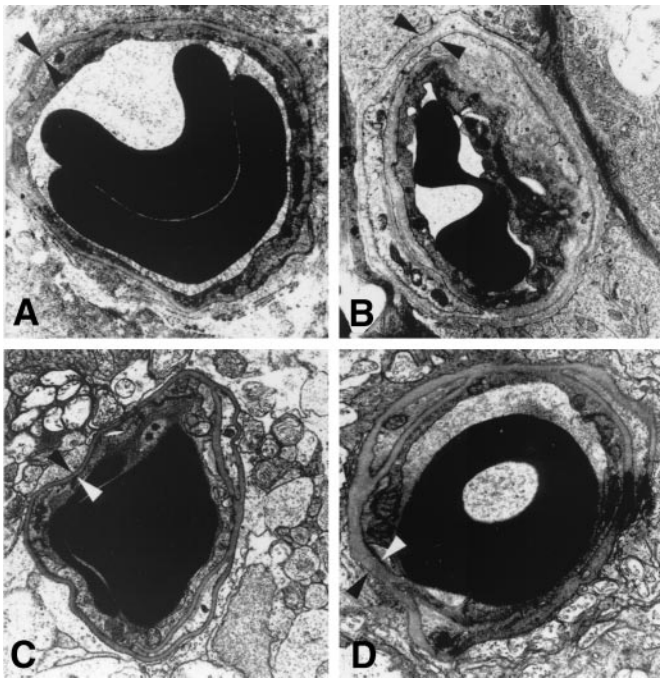

FIG. 3. Retinal capillary BM thickness. Representative transverse sections of retinal capillaries from the outer plexiform layer: control rat fed normal diet (*A*), galactose-fed rat (*B*), galactose-fed rat that received fibronectin antisense oligos treatment (*C*), and galactose-fed rat that received random oligos (*D*). Antisense treatment in the galactosemic rats specifically prevented thickening of the retinal capillary BM (arrow heads). Magnification, ×5,000. Figure shows significant reduction in BM thickening in retinas of eyes treated with fibronectin antisense oligos compared with retinas of untreated galactosemic rats.

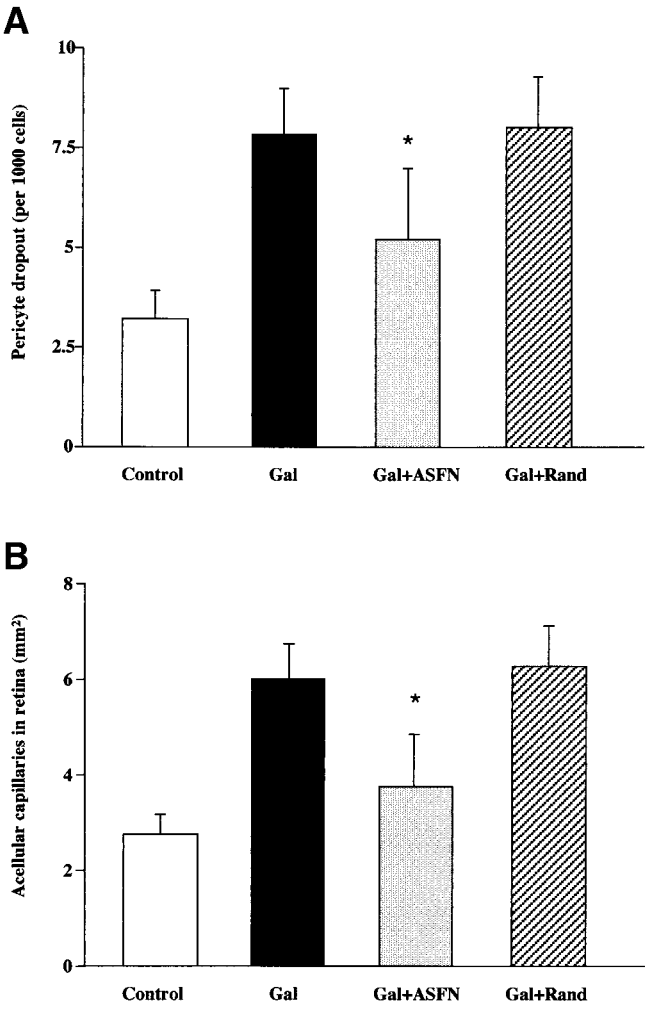

FIG. 4. *A*: Pericyte loss in retinal capillaries in control rats fed normal diet, galactose-fed rats, galactose-fed rats treated with fibronectin antisense oligos, and galactose-fed rats that received random oligos. Pericyte dropouts were significantly less in eyes treated with fibronectin antisense oligos compared with those of the galactosemic rats ($P < 0.04$) or galactosemic rats that received random oligos ($P < 0.03$). *B*: Antisense treatment in the galactosemic rats specifically inhibited the development of acellular capillaries ($n = 6$ for each group).

DISCUSSION

This study shows that the thickening of the retinal capillary BM in galactose-fed rats, an animal model of diabetic retinopathy, was significantly but partially prevented by intravitreal administration of phosphorothioate fibronectin antisense oligos targeted to the translation initiation site of the fibronectin transcript. Semiquantitative analysis showed significant downregulation of retinal fibronectin mRNA and protein expression in the fibronectin oligos–treated eyes compared with the untreated or random oligos-treated eyes. Sequence specificity and target specificity of the fibronectin antisense oligos were confirmed from several controls in this study. Fibronectin antisense oligos reduced only the fibronectin mRNA level and not the actin mRNA level; the retinal fibronectin expression remained unchanged in the "random" sequence oligos group; both fibronectin mRNA and protein level was reduced in a correlated manner ($r = 0.9$).

Several studies including ours had indicated that increased synthesis of BM components was associated with BM thickening (7–14); however, this is the first study that demonstrates a direct link between increased synthesis of fibronectin, a BM component, and the development of vascular BM thickening in diabetic retinopathy. This study also shows that the development of vascular lesions, pericyte loss, and acellular capillaries characteristic of diabetic retinopathy is at least in part dependent on alteration of the BM structure of the retinal capillaries. The finding that prevention of vascular BM thickening may have beneficial consequences toward retinal lesions in diabetic retinopathy underscores the importance of BM's role in maintaining vascular homeostasis and structural integrity of the retinal vasculature.

The thickening of the vascular BM remains the most prominent early histological lesion of diabetic microangiopathy and occurs in multiple organs. In this study, the validity of the antisense strategy was tested in an animal model of diabetic retinopathy that exhibits retinal microvascular lesions including vascular BM thickening (26). The antisense approach was specifically selected because its efficacy was demonstrated in vitro (21) and in vivo (22) toward reducing excess synthesis of ECM components and because our previous work had shown that upregulation of fibronectin synthesis occurred by 9 weeks of galactose-feeding, which provided us an important information regarding the initiation of antisense oligos treatment. Throughout the study period the presence of hyperhexosemia in the galactosemic rats treated with antisense oligos was confirmed; the glycemia level was similar between the galactosemic rats and the galac-

tosemic rats that received antisense treatment. Because diabetes or hyperhexosemia upregulates fibronectin gene expression in human and rat retinal microvessels (8,10,11), the maintenance of hyperhexosemia during the study period was necessary for testing the efficacy of the antisense strategy.

Studies have shown that the inner limiting membrane (ILM) is thickened in diabetes (13,27,28), which may suggest that increased synthesis of BM components may be a mechanism also operative in the thickened ILM. However, the consequence of thickened ILM in diabetic retinopathy is not well understood. From previous studies, we have observed that oligonucleotides delivered intravitreally initially localize in the ILM but then move through ILM and localize mostly in the capillary cells (22). The oligos would cause reduction of fibronectin accumulation in the retinal capillary BM by downregulating fibronectin overexpression in the vascular cells of retinal capillaries. Antisense oligos delivered intravitreally are unlikely to affect synthesis of soluble fibronectin, which is produced in the liver cells.

Studies with antisense oligos have indicated long-lasting effects. For example, a single intravenous injection of p65 phosphorothioate antisense oligonucleotides abrogated clinical signs of established intestinal inflammation in mice. The antisense effect lasted for as long as the animals were followed in that study (6 weeks) (29). In another study, a single intraluminal delivery of antisense cdc2 kinase and proliferating cell nuclear antigen oligonucleotides caused sustained inhibition of neointimal hyperplasia for 8 weeks (30). This study indicates that the effects of antisense oligonucleotides are long-lived in the retinal vascular cells and application of the antisense strategy may be achievable by therapeutic approaches that are not chronic in nature.

The feasibility of applying antisense oligos for treatment of ocular complications and diabetes is supported by several studies. Using antisense strategy, neovascularization in the retina was prevented by blocking vascular endothelial cell growth factor expression in a murine model of proliferative diabetic retinopathy (31). The antisense strategy has also been used to reduce ECM accumulation in glomerular capillaries that are targets of diabetic microangiopathy. In the rat glomerulus, the expression of transforming growth factor-β1 protein was markedly reduced by antisense oligos that prevented glomerular ECM expansion in experimental glomerulonephritis (32). Furthermore, Fomivirsen, an antisense oligos drug is currently available in the market for ocular use (33,34), indicating the therapeutic potential of the antisense strategy. Because vascular BM thickening is commonly present in the capillaries of patients with diabetic microangiopathy, our finding that retinal vascular lesions can be reduced by ameliorating BM thickening with the antisense oligos, indicates the potential usefulness of this antisense strategy in diabetic microangiopathy in general.

Abnormal levels of fibronectin have long been recognized in several diseases, including diabetes. Increase in tissue fibronectin of diabetic skin (35) and in the vitreous of patients with proliferative diabetic retinopathy (36) has been reported. The presence of fibronectin receptors in the newly formed retinal capillaries in proliferative diabetic retinopathy indicates that fibronectin possibly plays a key role in the structural arrangement of capillaries in proliferative diabetic retinopathy (37). Our recent finding indicates that altered fibronectin expression may contribute to increased vascular permeability (38). Overall, fibronectin appears to occupy a more important place in the pathogenesis of diabetic retinopathy than has been previously recognized.

Current studies are aimed at understanding whether the thickened BM plays an altered functional role in diabetic retinopathy. Recent observation indicates that high glucose-induced overexpression of fibronectin may contribute to increased endothelial cell permeability associated with blood retinal barrier breakdown in diabetic retinopathy (38). Overall, these results suggest that antisense oligos against fibronectin may provide a powerful interventive approach for reducing vascular lesions of diabetic retinopathy and studying the role of vascular BM thickening in the pathogenesis of diabetic microangiopathy.

ACKNOWLEDGMENTS

Work on BM project was supported by grants from the American Diabetes Association and the National Eye Institute, NIH (EY 11990-01A1), and in part by departmental grants from MA Lions Eye Research Foundation, Inc., and the Research to Prevent Blindness, Inc.

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