Original Article

Effect of Combined Antisense Oligonucleotides Against High-Glucose– and Diabetes-Induced Overexpression of Extracellular Matrix Components and Increased Vascular Permeability

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The effect of combined antisense oligonucleotides (AS-oligos) against overexpression of extracellular matrix (ECM) components, fibronectin, laminin, and collagen IV and on cell monolayer permeability was examined in rat microvascular endothelial cells (RMECs) grown in high glucose medium and on retinal vascular permeability in diabetic rats. RMECs grown in high glucose for 10 days and transfected with combined AS-oligos showed a significantly reduced fibronectin, laminin, and collagen IV protein level. In parallel studies, high-glucose–induced excess monolayer permeability was also reduced in RMECs transfected with the combined AS-oligos. Similarly, diabetic rats intravitreally injected with the combined AS-oligos and examined after 2 months of diabetes showed significant reduction in retinal fibronectin, laminin, and collagen IV expression. In addition, vascular permeability, as determined from extravasation of fluorescein isothiocyanate–BSA in the surrounding areas of the retinal capillaries, was partially reduced in the combined AS-oligos–treated diabetic retinas. Our results indicate that the combined AS-oligos strategy is effective in simultaneously reducing fibronectin, collagen IV, and laminin overexpression and reducing vascular leakage in the retinal capillaries of diabetic rat retinas. The findings suggest that abnormal synthesis of ECM components may contribute to vascular leakage in the diabetic retina. *Diabetes* 55:86–92, 2006

Increased synthesis of basement membrane components, fibronectin, collagen IV, and laminin is closely associated with vascular basement membrane thickening, a histological hallmark of diabetic retinopathy (1–5). In vivo and in vitro studies have shown that diabetes selectively increases basement membrane gene expression in retinal vascular cells and that the excess synthesis of basement membrane components contributes, at least in part, to the development of thickened vascular basement membranes (6–9). We have recently shown that the fibronectin antisense oligonucleotides (AS-oligos) reduce overexpression of basement membrane components and partially prevent the development of thickened vascular basement membrane in retinal capillaries of galactose-fed rats, an animal model of diabetic retinopathy, with beneficial consequences to histological lesions such as pericyte loss and acellularity (9). However, in order to completely prevent and reverse vascular basement membrane thickening, and regulate functional abnormalities, a more effective antisense strategy is necessary.

Breakdown of the blood-retinal barrier (BRB) is an early functional change associated with the retinal vasculature in diabetic retinopathy (10) that continues with the progression of the complication (11). The mechanism by which excess vascular permeability develops in diabetic retinopathy is not yet established. However, it is clear that although the excess permeability of macromolecules may involve increased paracellular and/or transcellular pathways, ultimately the macromolecules after crossing the cellular layer must cross the basement membrane in order to exhibit vascular permeability.

Thickening of capillary basement membrane has been widely observed in diabetic humans and animals (12) and is the most prominent histological abnormality associated with the capillaries in the diabetic retina. A study examining glomerular basement membrane width in kidney biopsies and analyzing retinal complications in diabetic patients suggested a link between the severity of retinopathy and increased basement membrane thickening (13). Despite the striking histological changes in the diabetic retinal vasculature, functional consequences of these changes have remained elusive.

Several studies have suggested that the efficacy of the combined AS-oligos may be a potentially more powerful strategy than the single AS-oligo approach. Potential additive effects of multitarget AS-oligo strategy have been documented (14–16). Studies have shown that the combined application of two AS-oligos inhibited the growth of tumors and caused a remarkable increase in the survival of treated mice (17). Because the vascular basement membrane is a multimeric structure, it is well suited for applying the combined AS-oligos strategy. Having already developed the individual AS-oligos, capable of indepen-

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AS-oligo, antisense oligonucleotide; BRB, blood-retinal barrier; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; Ran-oligo; random oligonucleotide; RMEC, rat microvascular endothelial cell; STZ, streptozotocin; VEGF, vascular endothelial growth factor.

dently reducing fibronectin, collagen IV, or laminin expression, in this study, we investigated using a combined AS-oligos approach whether the altered basement membrane composition plays a role in excess vascular permeability associated with diabetic retinopathy.

RESEARCH DESIGN AND METHODS

Cell culture. Rat microvascular endothelial cells (RMECs) positive for von Willebrand factor were used in this study (18–20). Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, antibiotics, and antimycotics. To determine the effect of high glucose on fibronectin, collagen IV, and laminin protein levels, these cells were grown in normal (5 mmol/l) or high (30 mmol/l) D-glucose medium for 10 days to confluency. Cells were transfected at subconfluency at day 7, with AS-oligos designed to reduce extracellular matrix (ECM) expression in these cells. Seventy-two hours posttransfection, cells were harvested and protein isolated for Western blot analysis.

Animals. Sprague-Dawley male rats weighing ~200 g were used in this study. Streptozotocin (STZ) was injected into tail veins at 55-mg/kg dose to induce diabetes. The glucose concentration in blood and urine was checked after 3 days following STZ injection to confirm diabetes status in the animals. Glycohemoglobin levels were measured in each animal at the time of death using a commercially available kit (Glyc-Affin; Pierce, Rockford, IL). A total of 28 rats were randomly divided into four groups consisting of 7 rats in each group: nondiabetic control rats, STZ-induced diabetic rats, STZ-induced diabetic rats treated with combined AS-oligos, or STZ-induced diabetic rats treated with random oligonucleotides (Ran-oligos). A cocktail containing a 3-μmol/l dose for each of the three AS-oligos (against fibronectin, collagen IV, and laminin) was administered monthly for 1 or 2 months by intravitreal injection (9,20,21). As a control for specificity of antisense effect, rats were also intravitreally injected with 3 μmol/l Ran-oligos monthly. Briefly, under deep anesthesia, AS-oligos or Ran-oligos was injected into vitreous using a 30-gauge needle. About 0.5 mm posterior to the limbus, the needle was inserted into the vitreous at an angle of ~40°. No nonspecific inflammatory reactions such as fibrosis strands or increased vitreal scattering were observed in the vitreous after AS-oligos or Ran-oligos injection. One and 2 months post–AS-oligos treatment, animals were killed, retinas were isolated, and retinal fibronectin, collagen IV, and laminin protein levels were determined by Western blot analysis. Retinal vascular permeability was determined from digital images showing fluorescence intensities in retinal whole mounts and cryosections obtained after fluorescein isothiocyanate (FITC)-BSA (50 mg/kg) tail vein injection immediately before the rats were killed. At the time of death, blood was collected and was centrifuged at 2,000*g* for 10 min, and the plasma was assayed for fluorescence in a spectrophotometer with excitation at 433 nm and emission at 455 nm. All animals were used according to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research.

Antisense oligonucleotides treatment. Sixteen-, 17-, or 19-mer phosphorothioate AS-oligos (5′-CCTGAGCATCTTGAGTC-3′, 5′-TGAGCCGGGGC CCCATGGT-3′, 5′-CCGTCATCCCGCCGTG-3′) encompassing the translation initiation site of the fibronectin, collagen IV, and laminin transcripts were used to reduce fibronectin, collagen IV, and laminin expression in RMECs and rat retinas. In studies using cell cultures, 0.4 μmol/l of each of the three AS-oligos were transfected with 8 μmol/l lipofectin for 72 h into subconfluent cells. In case of intravitreal delivery of combined AS-oligos, the oligo-polymer complexes were produced by mixing 3 μmol/l AS-oligos with 6 μmol/l polyoxyethylene-polyspermine and used within an hour after the preparation. Ran-oligos were used as negative controls in both in vitro and in vivo studies.

Western blot analysis. RMECs and retinas were isolated and incubated in 25 mmol/l Tris, pH 7.4, containing 1 mmol/l EDTA, 1 mmol/l phenymlmethylsulfonyl fluoride, and 0.1% Triton X-100. Western blots were performed with 25 μg protein/lane and after electrophoresis transferred onto nitrocellulose membrane according to Towbin's procedure (22). After blocking with 5% fat-free milk in tribromosalicylanilide, the membrane was incubated with the blocking solution containing either rabbit anti-fibronectin, anti–collagen IV, or anti-laminin antibody (1:500, 1:500, 1:1,000, respectively) overnight. After washing, the membrane was incubated with anti-rabbit antibody conjugated with horseradish peroxidase (1:15,000) for 1 h. The Immuno-Star Chemiluminescent Protein Detection System was used to detect protein levels of fibronectin, collagen IV, and laminin. Densitometric analysis of the luminescent signal was performed at nonsaturating exposures with a laser scanning densitometer and NIH Image software. To determine β-actin signals, the membranes exposed to fibronectin, laminin, and collagen IV antibodies were stripped with a buffer containing 62.5 mmol/l Tris, pH 6.8, 2% SDS, and 100 mmol/l 2-mercaptoethanol and reused with β-actin antibody solution (1:2,500). After washing, the membranes were incubated with anti-mouse antibody conjugated with horseradish peroxidase (1:15,000) and applied to the Immuno-Star Chemiluminescent substrate and exposed to X-ray film.

In vitro permeability assay. In parallel to the aforementioned cell culture protocol, RMECs grown on inserts of 24-transwell plates in normal or high glucose medium were transfected with combined AS- or Ran-oligos on day 7. In vitro permeability assay was performed in confluent monolayers by adding FITC-dextran at a concentration of 1 mg/ml to the upper chamber and determining the passage of FITC-dextran into the lower chamber of the transwells. Aliquots taken from the lower chamber were measured in a spectrophotometer at 492 nm.

Cell survival and proliferation assay. Dose-dependent effect of AS-oligo on cell survival or proliferation was examined in RMECs grown in high glucose medium. Increasing concentration of 0.04, 0.1, 0.4, and 1.0 μmol/l for each of the three AS-oligos (fibronectin AS-oligo, collagen IV AS-oligo, and laminin AS-oligo) or similar concentrations of Ran-oligos were transfected into semiconfluent cells grown in high glucose medium and assayed after 72 h. In each experiment ($n = 6$), an equal number of cells were seeded at the start of the experiment. Cells were counted in duplicate in a Coulter counter (Coulter Electronics, Hialeah, FL) and randomly verified with a hemocytometer.

Measurement of BRB permeability in vivo. To measure BRB permeability, we followed the previously published method of Antonetti et al. (24,25) with slight modification in this study. Briefly, tail vein injections with 50 mg/kg FITC-BSA were performed in rats with 1 or 2 months of diabetes. Twenty minutes after the injection, the animals were killed and the eyes were enucleated and immediately placed in 10% formaldehyde. At the time of death, blood was collected and the plasma assayed for fluorescence. The fixed retinas were sectioned (8 μm thick), and fluorescence intensities of at least 10 vertical sections of vessels were randomly identified from the digital images and measured for fluorescence by assessing the mean intensities of vessels and leakage areas (10^4 pixels) by NIH Image Analysis program. Retinal whole mounts were prepared from one retina from each animal, and the pattern of vascular leakage was observed under fluorescence microscope. The average retinal fluorescence intensity was normalized to plasma fluorescence intensity of each animal taken at the time of death. Data are shown as mean times area divided by plasma fluorescence intensity.

Statistical analysis. Data are expressed as means ± SD. Comparisons between groups were made using ANOVA followed by Student's t test and Mann-Whitney U test, with a level of $P < 0.05$ as significant.

RESULTS

Effect of combined AS-oligos on fibronectin, collagen IV, and laminin protein levels in vitro. RMECs grown in high glucose medium for 10 days and subsequently analyzed for protein expression exhibited increased protein level for fibronectin (174 ± 32% of control, $P < 0.01$, $n = 4$), collagen IV (156 ± 23% of control, $P < 0.01$, $n = 7$), and laminin (128 ± 18% of control, $P < 0.01$, $n = 7$) compared with cells grown in normal medium (Table 1, Fig. 1). Cells grown in high glucose medium and subsequently transfected with combined AS-oligos showed reduced protein level for the three ECM components, fibronectin (60 ± 21% of control, $P < 0.01$, $n = 4$), collagen IV (86 ± 24% of control, $P < 0.01$, $n = 7$), and laminin (96 ± 29% of control, $P < 0.05$, $n = 7$) when compared with cells grown in high glucose medium (Table 1, Fig. 1). Cells grown in high glucose medium and subsequently transfected with Ran-oligos produced no significant reduction in protein levels for fibronectin, collagen IV, or laminin (Table 1, Fig. 1).

Effect of high glucose, combined AS-oligos, and Ran-oligos on monolayer permeability in vitro. Cells grown in high glucose medium showed increased monolayer permeability compared with cells grown in normal medium (156 ± 41% of control, $P < 0.05$, $n = 11$). Transfection with combined AS-oligos significantly reduced monolayer permeability compared with cells grown in high glucose medium (90 ± 26% of control vs. 156 ± 42% of control, $P < 0.01$, $n = 11$) or cells grown in high glucose medium and transfected with Ran-oligos (90 ± 26% of control vs. 138 ± 37% of control, $P < 0.05$, $n = 11$). Cells

TABLE 1
Effect of combined AS-oligos against expression of ECM components, fibronectin, collegen IV, and laminin in cells grown in high glucose medium compared with cells grown in normal medium or cells transfected with Ran-oligos

ECM gene	Normal	High glucose	High glucose + antisense	High glucose + random
Fibronectin (n = 4) (%)	100 ± 29	174 ± 32*	60 ± 21†	167 ± 31
Collagen IV (n = 7) (%)	100 ± 15	156 ± 23*	86 ± 24†	124 ± 17
Laminin (n = 7) (%)	100 ± 16	128 ± 18*	96 ± 29†	123 ± 17

Date are means ± SD (% of control). *$P < 0.05$ compared with normal; †$P < 0.05$ compared with high glucose.

grown in high glucose medium transfected with Ran-oligos produced no significant reduction in monolayer permeability compared with cells grown in high glucose medium (138 ± 37% of control vs. 156 ± 42% of control, n = 11). As expected, a significant difference in monolayer permeability was observed between high glucose cells transfected with Ran-oligos and cells grown in normal medium (138 ± 37% of control vs. 100 ± 29% of control) (Fig. 2*A*).

Effect of low-dose AS-oligos against ECM component expression and its effect on cell survival or proliferation. A dose-response curve was established with increasing concentration for each of the three AS-oligos, which included the 0.4-μmol/l dose used in this study for the in vitro experiments. Cell counts at varying doses showed practically no difference, indicating that the AS-oligo dose used in this study did not affect cell survival or proliferation of RMECs. Additionally, this indicated that the transfection process itself had no effect on cell survival or proliferation (Fig. 2*B*).

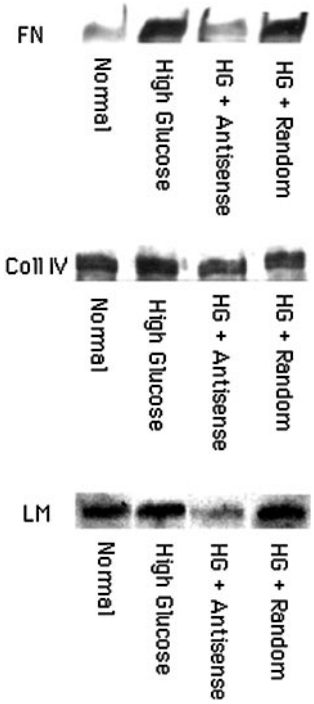

FIG. 1. Western blot analysis of fibronectin, collagen IV, and laminin protein levels in cells grown in normal, high glucose, high glucose transfected with AS-oligos, and high glucose transfected with Ran-oligos mediums. Cells grown in high glucose medium showed increased fibronectin, collagen IV, and laminin expression compared with those grown in normal medium. Cells grown in high glucose medium and transfected with AS-oligos showed simultaneous reduction in fibronectin, collagen IV, and laminin expression compared with cells grown in high glucose medium.

Effect of combined AS-oligos strategy on fibronectin, collagen IV, and laminin protein levels in vivo. At the time of death, the glycohemoglobin levels confirmed the presence of hyperhexosemia in diabetic rats compared with control rats (12.2 ± 3.6 vs. 4.6 ± 0.5%, $P < 0.01$). After 1 month of diabetes (n = 7), no significant change in fibronectin, laminin, and collagen IV protein levels was observed (Table 2). After 2 months of diabetes (n = 7), retinal fibronectin, collagen IV, and laminin protein level was significantly increased (195 ± 38% of control, 191 ± 48% of control, and 183 ± 54% of control, respectively) compared with those in control nondiabetic rats (n = 7). When diabetic rats were treated with combined AS-oligos and examined 2 months posttreatment (n = 7), the fibronectin, collagen IV, and laminin protein level was significantly reduced (147 ± 29% of control vs. 195 ± 38% of control, 146 ± 28% of control vs. 191 ± 48% of control, and 128 ± 47% of control vs. 183 ± 54% of control, respectively, compared with those of diabetic retinas, $P < 0.05$). Treatment with Ran-oligos (n = 7) showed no effect after 2 months posttreatment (201 ± 46% of control vs. 195 ± 38% of control, 202 ± 41% of control vs. 191 ± 48% of control, and 184 ± 31% of control vs. 183 ± 54% of control, respectively) (Table 2, Fig. 3). All densitometric values for Western blot analysis have been corrected with corresponding β-actin signals (Table 2, Fig. 3).

Effect of diabetes, combined AS-oligos, and Ran-oligos on retinal vascular permeability in vivo. After 1 month of diabetes, only in some areas of retinas, an indication of extravasation was apparent from increased fluorescence intensity in the retinal vessels compared with control (354 ± 71% of control, $P < 0.01$). At the 1-month time point, the excess permeability was not affected by AS-oligo transfection. No significant difference in the degree of fluorescence intensity was observed when AS-oligos–treated retinas were compared with Ran-oligos–treated or untreated diabetic retinas (204 ± 34% of control vs. 354 ± 71% of control and 370 ± 181% of control vs. 354 ± 71% of control, respectively). These results indicate that factors other than overexpression of ECM components may contribute to increased vascular permeability in early diabetic retinopathy. After 2 months of diabetes, fluorescence intensities in the retinal vessels and in several areas surrounding the retinal vessels of the diabetic retinas were significantly increased compared with control retinas (337 ± 64% of control, $P < 0.01$) (Figs. 4, 5, and 6*A*). In the images of whole-mount retinas of diabetic rats, some areas showed diffused "patches" at the site of intense vascular leakage (Fig. 4). In the diabetes plus Ran-oligo–treated retinas, some areas of nonperfusion and sacular aneurismal-like features similar to retinal capillaries in the diabetes plus Ran-oligo group were noticed (Fig. 4). We have observed occasional background fluorescence in the

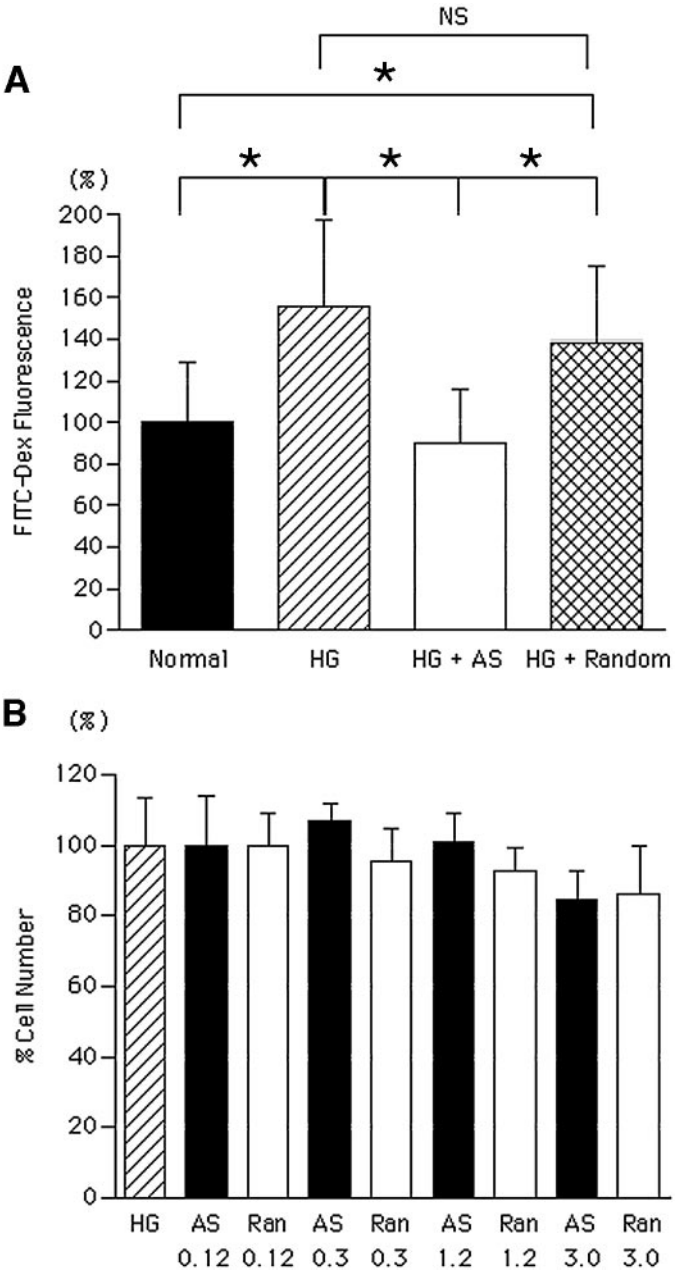

FIG. 2. Graphical illustration of aggregate results of in vitro permeability assays showing a dose-dependent effect of AS-oligos on RMEC survival and proliferation. *A*: Cells grown in high glucose medium showed increased monolayer permeability compared with cells grown in normal medium. Cells grown in high glucose medium and transfected with combined AS-oligos showed reduced monolayer permeability compared with cells grown in high glucose medium. Cells grown in high glucose medium and transfected with Ran-oligos showed no reduction in monolayer permeability compared with cells grown in high glucose medium only. *B*: Dose ranging from 0.12 to 3.0 μmol/l of AS-oligos, which encompasses the dose of 0.4 μmol/l used in the study, did not affect cell survival or proliferation of RMECs grown in high glucose medium. *$P < 0.05$. HG, high glucose; AS, AS-oligos; Ran, Random; NS, not significant.

whole-mount retinal sections (Fig. 4, *DM*) that may be associated with choroidal leakage. However, at this time, we are unable to ascribe this signal to choroidal leakage (Fig. 4). In the cryosections, we did not observe any background fluorescence (Fig. 5). Therefore, it is unlikely that choroidal leakage may contribute to the measurements. Also, fluorescence intensity was generally measured from areas that had no background fluorescence. In

TABLE 2
Effect of combined AS-oligos against expression of ECM components, fibronectin, collegen IV, and laminin in retinas of diabetic rats

	Normal	Diabetes	Antisense	Random
1 month				
Fibronectin	100 ± 7	124 ± 54	94 ± 16	109 ± 22
Collagen IV	100 ± 21	155 ± 87	101 ± 38	163 ± 108
Laminin	100 ± 31	127 ± 59	96 ± 21	114 ± 18
2 months				
Fibronectin	100 ± 6	195 ± 38*	147 ± 29†	201 ± 46
Collagen IV	100 ± 14	191 ± 48*	146 ± 28†	202 ± 41
Laminin	100 ± 10	183 ± 54*	128 ± 47†	184 ± 31

Data are means ± SD (% of control). *$P < 0.05$ compared with control retinas; †$P < 0.05$ compared with diabetic retinas.

combined AS-oligos–treated diabetic retinas, the degree of fluorescence in and around the retinal vessels was significantly decreased compared with untreated diabetic retinas (158 ± 23% of control vs. 337 ± 64% of control, $P < 0.05$) (Figs. 4–6*A*) or compared with Ran-oligos–treated diabetic retinas (158 ± 23% vs. 307 ± 45% of control, $P < 0.05$). A reference point for the evaluation of the excess permeability associated with the increased ECM expression is indicated in Fig. 6*B*.

DISCUSSION

To evaluate whether combined AS-oligos simultaneously reduce three ECM components, we first tested fibronectin, collagen IV, and laminin protein levels in cells transfected with combined AS-oligos in vitro and, as a functional

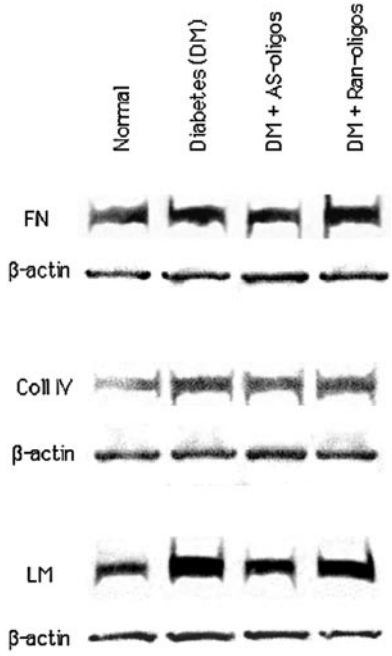

FIG. 3. Western blot analysis of fibronectin, collagen IV, and laminin protein levels in normal and diabetic retinas, diabetic retinas treated with combined AS-oligos, and diabetic retinas treated with Ran-oligos. In retinas of rats with 2 months of diabetes, fibronectin, collagen IV, and laminin protein expression was significantly increased compared with normal retinas. In diabetic retinas treated with combined AS-oligos, fibronectin, collagen IV, and laminin protein expression was significantly decreased compared with those in diabetic retinas. Retinas treated with Ran-oligos showed no effect. All densitometric values for Western blot analysis were normalized with β-actin signals.

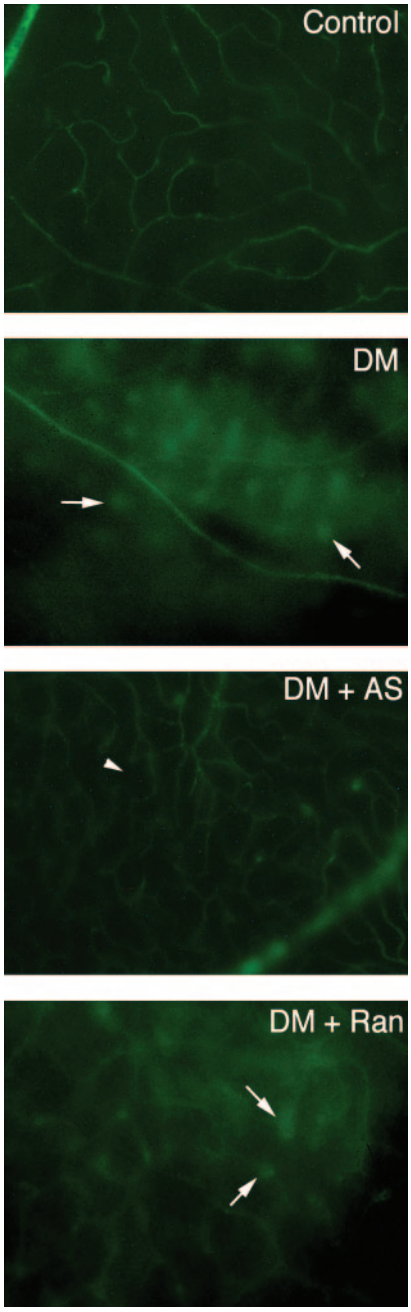

FIG. 4. Representative areas showing FITC fluorescence in vessels of whole-mount retinas. Areas of intense FITC leakage from extravasation were observed in the diabetic (DM) retinas compared with control nondiabetic retinas. Also, in the diabetic retinas, some areas showed diffused "patches" at the site of intense vascular leakage (arrows). In the diabetic rat retinas, combined AS-oligos (AS) treatment partially but significantly reduced areas of extravasation associated with excess vascular permeability. Ran-oligos (Ran) treatment showed no effect on retinal vascular permeability. Nonperfused vessel (arrowhead). Magnification 200×.

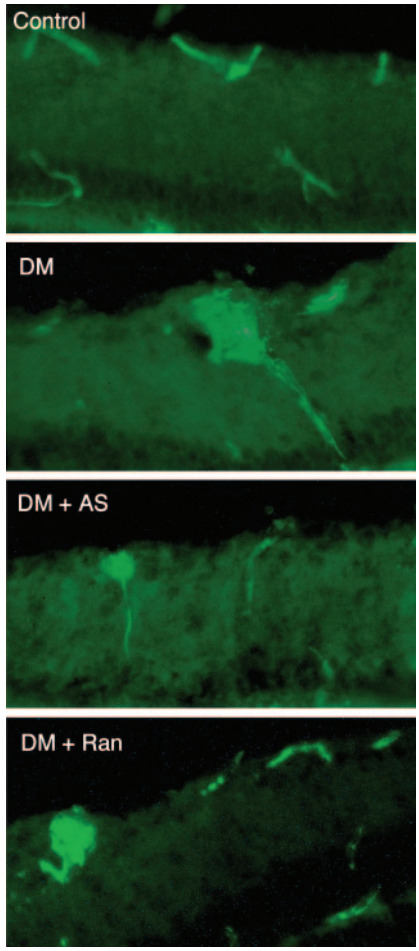

FIG. 5. Representative images of retinal cryosections from nondiabetic control rats, diabetic (DM) rats, diabetic rats injected with combined AS-oligos (AS), and diabetic rats injected with Ran-oligos (Ran). In diabetic retinas, extravasation of FITC in and around the vessels was significantly increased compared with those in the nondiabetic control retinas. Combined AS-oligos–treated retinas showed reduction in vascular leakage compared with those in untreated diabetic retinas or Ran-oligos–treated diabetic retinas. Magnification 200×.

assay, examined cell monolayer permeability in RMECs exposed to high glucose and transfected with combined AS-oligos. The protein expression of all three ECM components showed significant and simultaneous reduction in cells transfected with the combined AS-oligos. In addition, cell monolayer permeability was significantly reduced in the combined AS-oligos–transfected cells. In vivo studies in which intravitreal injection of combined AS-oligos was performed in diabetic rats showed significant downregulation of retinal fibronectin, collagen IV, and laminin expression with partial reduction in retinal vascular permeability. These results indicate that the translation initiation site for each of the three ECM components is an effective target site for simultaneous inhibition of expression of the three ECM components and that the excess vascular permeability in the diabetic rat retinas is, at least in part, due to altered composition of the retinal vascular basement membrane.

Several laboratories including ours have previously demonstrated increased synthesis of basement membrane components in cultured endothelial cells grown in high glucose medium (26–28), in tissues of diabetic rats (6), and in retinal vessels of patients with diabetes (7,8). These studies have established that both high glucose and diabetes increase synthesis of fibronectin, collagen IV, and laminin (6–8,26,27,29–34). However, the functional consequence of the altered synthesis of ECM components has remained unclear. Molecular and biochemical changes, together with several morphological changes, may account for the characteristic breakdown of the BRB in diabetic retinopathy. Studies have shown alteration in the endothelial cytoplasm with resultant transendothelial channels (35), changes in the intercellular tight junctions

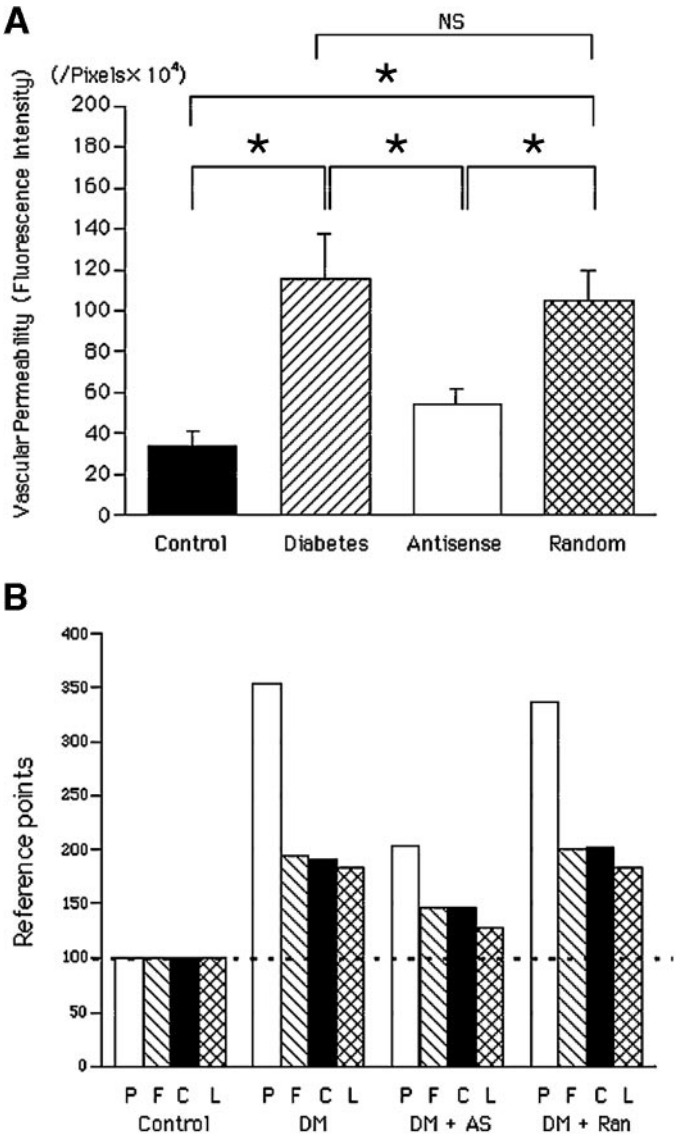

FIG. 6. Graphical illustration of aggregate results of in vivo permeability assays. *A*: In retinas of 2-month diabetic rats, the areas of FITC extravasation were significantly increased compared with those in control retinas. Combined AS-oligos–treated retinas showed partially but significantly reduced vascular leakage compared with the untreated diabetic retinas. Ran-oligo treatment showed no effect on retinal vascular permeability. Data shown are means ± SE. *B*: Reference points for the evaluation of excess permeability in relation to increased synthesis of ECM components. *$P < 0.05$. P, permeability; F, fibronectin; C, collagen IV; L, laminin; NS, not significant.

and the composition of the tight junction components ZO-1 (36), occludin (37), and claudin (38) as well as the formation of gaps between cells of the BRB. While it is possible that these factors may influence the breakdown of the BRB during early changes in diabetic retinopathy (10), our finding indicates that altered ECM composition may influence factors such as basement membrane remodeling and also contribute to BRB breakdown in diabetic retinopathy.

The remodeling of ECM is a dynamic process intimately associated with the synthesis and degradation of its components. In the diabetic retinal capillaries, significant remodeling takes place, including endothelial thinning, shape changes, loosening of vascular sheaths, and basement membrane thickening. These changes may be closely associated with increased vascular permeability in diabetic rat retinas (39). In diabetic retinopathy, upregulation of basement membrane component synthesis can result in an altered ratio of basement membrane components and in turn may impact basement membrane molecular sieve properties and the number of ionic sites on the basement membrane. Leakages have been documented in glomerular basement membrane with altered ionic sites (40). It is still incompletely understood as to how changes in the composition of ECM components might relate to increased vascular permeability. However, it is evident that remodeling of the basement membrane plays an essential role in altered vascular permeability.

Although basement membrane remodeling is an important factor in vascular permeability, and alteration of this process may profoundly affect vascular leakage in diabetic retinopathy, there are other factors that may play a role in excess vascular permeability. For example, vascular endothelial growth factor (VEGF) and tumor necrosis factor-α come into play early in diabetes. Studies using animal models of diabetes have shown that in the early stages of diabetic retinopathy, tumor necrosis factor-α may play a role in inducing increased vascular permeability (41). Using tumor necrosis factor-α inhibitor, etanercept, Joussen et al. (41) achieved a 50% reduction in vascular permeability in 1-week-diabetic rats. The involvement of VEGF in early stages of human diabetic retinopathy has also been reported. Immunofluorescence staining performed in human autopsy eyes with nonproliferative diabetic retinopathy and without retinal nonperfusion showed increased VEGF expression compared with nondiabetic controls (42,43). Also, VEGF injected into primate eyes produced some microaneurysms and vascular leakage that are signs of nonproliferative diabetic retinopathy (44). These studies together with our current findings suggest that in early stages of diabetes, not one but several factors are operative, including growth factors and cytokine activity, and basement membrane remodeling that may contribute to the development of excess permeability.

In this study, we observed that the combined AS-oligos approach is potentially a more effective strategy than the single oligo strategy against increased synthesis of basement membrane components in diabetic retinopathy. Consistent with these results, potential additive effects of the multitarget oligos strategy have been documented in other studies (14–16). In one study, systemic administration of antisense Bcl-xL and Bcl-2 oligos in mice bearing tumors significantly delayed time in the development of tumor progression (16). In another study, the combined application of two AS-oligos inhibited the growth of tumors and caused a remarkable increase in the survival of treated mice (17). Although, in these studies, the combined AS-oligos approach was administered in nonocular tissues, the additive effect against the multiple targets was evident. To the best of our knowledge, findings from this study are the first demonstration that combined AS-oligos targeting three components has a cooperative activity in the retinal vascular cells.

The cause of excess vascular permeability in diabetic retinopathy remains unresolved, with some investigators reporting that BRB breakdown is mediated by altered membrane permeability and increased vesicle formation (45,46) and others reporting altered junction protein expression (24). While these studies primarily take into account the effect of cellular alterations on vascular permeability, findings from this study suggest that diabe-

tes-induced changes in the composition of the basement membrane may affect vascular permeability in the retina.

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