Oncofetal Fibronectin in Diabetic Retinopathy

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Purpose. Imbalance between extracellular matrix protein synthesis and degradation is a key feature of diabetic retinopathy. Fibronectin, a predominant constituent of the extracellular matrix, has been shown to undergo alternative splicing to produce embryonic isoforms in various pathologic conditions, such as fibrotic diseases and tumorigenesis. Two such isoforms, oncofetal fibronectin variants that are characterized by the inclusion of the oncofetal domains A and B, were the focus of the present study.

Methods. The expression of oncofetal fibronectin variants was determined in human vitreous samples obtained from patients undergoing vitrectomy for proliferative diabetic retinopathy and nondiabetes-associated ocular conditions such as macular hole. In addition, an animal model of chronic diabetes and cultured endothelial cells was used to elucidate the mechanistic basis for this aberrant expression of oncofetal fibronectin.

Results. Expression of fibronectin containing the oncofetal domain B was upregulated in the vitreous of patients with diabetic retinopathy.

Conclusions. Use of a well-established animal model of chronic diabetic complications and cultured endothelial cells showed that diabetes-induced upregulation of oncofetal fibronectin is, in part, dependent on hyperglycemia-induced transforming growth factor-β1 and endothelin-1. Furthermore, the data suggest that oncofetal fibronectin is involved in endothelial cell proliferation. (*Invest Ophthalmol Vis Sci.* 2004;45:287–295) DOI:10.1167/iovs.03-0540

Diabetic retinopathy is the leading cause of blindness in the working population.[1,2] The characteristic retinal changes associated with long-standing diabetes include capillary basement membrane thickening (CBMT).[3–5] Increased extracellular matrix (ECM) protein deposition is instrumental in CBMT and is a structural hallmark of diabetic retinopathy.[3–5] One of the major alterations observed in CBMT is the accumulation of a predominant ECM protein fibronectin (FN).[5] We and others have demonstrated upregulation of FN mRNA in target organs of late complications in both animal and human diabetes.[6–7]

FN plays key roles in various cellular events that include cell migration and proliferation of fibroblasts and endothelial cells.[8,9] Two forms of FN are present, circulating plasma FN and locally synthesized cellular FN. The plasma form of FN is synthesized by hepatocytes, and the cellular form is synthesized by several cells, including endothelial cells.[8–10] mRNA of FN, encoded by a single gene with 50 exons, is processed by alternative splicing. Three sites for pre-mRNA processing have been identified: extra domain A (EDA), extra domain B (EDB), and type III homology connecting segment (IIICS).[8,9,11] In plasma FN, both EDA and EDB are excluded (EDA− and EDB−). Whereas, cellular FN can have EDA and/or EDB included or excluded, depending on the cellular and extracellular contexts. The IIICS region, also known as the variable region, contains multiple splice sites within, producing five IIICS variants in humans (IIICS-0, IIICS-120, IIICS-95, IIICS-89, and IIICS-64). In addition to pre-mRNA processing, the FN molecule can also undergo posttranslational modifications, notably *O*-linked glycosylation in the IIICS region.[12] Thus, differential pre-mRNA processing and posttranslational modifications can lead to 20 different FN isoforms.[8,9,11]

Inclusion of EDA is believed to be distinctive in cellular FN. Although undetectable in normal adult liver, an upregulation of EDA^+ FN occurs in liver fibrosis.[13] In addition, this variant is overexpressed in proliferating tissues such as embryos and tumors.[14–16] EDB^+ FN is also expressed only in embryonic and tumor tissues and has recently been proposed as an angiogenic marker.[16–19] These two isoforms, EDA^+ FN and EDB^+ FN, along with the *O*-glycosylated form of FN are referred to as oncofetal FN because of their upregulation during development and cellular transformation.

Accumulating evidence indicates that growth factors, such as transforming growth factor (TGF)-β, are involved in preferential expression of oncofetal FN in various tissues during remodeling and wound healing.[13,20] In addition, cultured cells also show differential FN expression in response to stimulation with TGF-β.[21,22] Because alteration of both TGF-β1, the predominant TGF-β isoform in diabetic complications, and endothelin (ET)-1 may be a factor in mediating increased ECM deposition in organs targeted by chronic diabetic complications including the retina,[6,23] we sought to investigate differential FN expression in the milieu of diabetes.

In the present study, we examined vitreous samples from patients undergoing vitrectomy for proliferative diabetic retinopathy (PDR) or non–diabetes-related ocular conditions such as macular hole. To test whether hyperglycemia-induced TGF-β1 and ET-1 are involved in differential FN expression, we used a well-established animal model of chronic diabetes. In addition, we evaluated the role of TGF-β1 and ET-1 in cultured endothelial cells, the primary target of hyperglycemia-induced dysfunction and the major contributors of FN in the retinal vasculature.

Methods

Human Vitreous Samples

All vitreous samples were obtained from patients during surgery after approval by Ethical Committee at University of Western Ontario and London Health Sciences Center, London, Ontario, and in accordance with the guidelines of the Declaration of Helsinki for research involving human tissue. The samples were divided into two groups. One group comprised samples from patients with PDR (n = 18; 7 women, 11 men; mean age ± SD, 58.1 ± 13.3 years) and the second group comprised samples from nondiabetic patients (n = 6; 5 women, 1 man; mean age ± SD, 69.6 ± 8.91 years). The samples were centrifuged (12,000*g*, 10 minutes; 4°C), and the pellet was used for RNA isolation and real-time quantitative RT-PCR. In PDR, vitreous predominantly contains endothelial cells from abnormal new blood vessels

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TABLE 1. Primer Sequences for Real-Time RT-PCR

Gene	Species	Sequence (5′ → 3′)	Size (bp)	Ref./Accession Number/(Nucleotides)
18S rRNA	Human/Rat	GTAACCCGTTGAACCCCATT CCATCCAATCGGTAGTAGCG	153	Ref. 28
EDB^+ FN	Human/Rat	CCGCCATTAATGAGAGTGAT AGTTAGTTGCGGCAGGAGAAG	129	X07717 (1483–1612) NM019143.1 (4109–4238)
EDA^+ FN	Human	CATTCACTGATGTGGATGTC CAGTGTCTTCTTCACCATCA	143	X07718 (1269–1412)
	Rat	GACTGTGTACTCAGAACCCG ACAGGGTGACCTACTCAAGC	115	NM019143.1 (5462–5578)
Total FN	Human	GATAAATCAACAGTGGGAGC CCCAGATCATGGAGTCTTTA	184	X02761 (100–284)
	Rat	CCAGGCACTGACTACAAGAT CATGATACCAGCAAGGACTT	145	NM019143.1 (6100–6245)
TGF-β1	Human	GCCCACTGCTCCTGTGACA CGGTAGTGAACCCGTTGATGT 6FAM-CAGGGATAACACACTGC-MGBNFQ	63	NM000660.1 (1505–1568)
	Rat	GTAGCTCTTGCCATCGGG GAACGTCCCGTCAACTCG	216	Ref. 29
ET-1	Human	AAGCCCTCCAGAGAGCGTTAT CCGAAGGTCTGTCACCAATGT 6FAM-TGACCCACAACCGAG-MGBNFQ	58	NM01955 (795–853)
	Rat	GCTCCTGCTCCTCCTTGATG CTCGCTCTATGTAAGTCATGG	500	NM012548.1 (342–842)

that grow in the plane anterior to the normal retinal vessels and extend into the vitreous.[24] Samples with clear indication of vitreal hemorrhage were excluded from the study because of the presence of RNA from blood cells.

Animal Model of Chronic Diabetes

Male Sprague-Dawley rats (Charles River Canada Ltd., St. Constant, Quebec, Canada), weighing 200 to 250 g, were made diabetic by a single intravenous injection of streptozotocin (65 mg/kg).[25,26] Hyperglycemia was confirmed by blood glucose measurement (Surestep/Lifescan Burnaby, British Columbia, Canada). Age- and sex-matched control animals received citrate buffer.

Diabetic rats were divided into two groups, diabetics (DM) and diabetics receiving the dual ET-receptor antagonist bosentan (DM-B; courtesy of Martine Clozel; Acetelion Ltd., Allschwill, Switzerland). Bosentan was administered by oral gavage (100 mg/kg per day).[25] Animals were monitored for glucosuria and ketonuria (Uriscan Gluketo; Yeong Dong Co., Seoul, Korea) and received small daily doses (0.1–3.0 U) of ultralente insulin (Novo Nordisk, Princeton, NJ) to prevent ketoacidosis. After 3 months of treatment, rats were killed, and retinal tissues were obtained. In addition, blood was obtained before death for measurement of glycated hemoglobin (Glycotest; Pierce, Rockford, IL). All animal care adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The University of Western Ontario Council on Animal Care formally approved all experimental protocols.

Endothelial Cells

Human microvascular endothelial cells (Clonetics, Walkersville, MD) were cultured with endothelial growth medium (Clonetics) as previously described.[27,28] Subconfluent cells were incubated with either 5 mM D-glucose (control) or 25 mM D-glucose (high glucose; HG). Cells in 5 mM glucose were also treated with 1 ng/mL recombinant human TGF-β1 (Sigma-Aldrich, St. Louis, MO) or 5 nM ET-1 (Peninsula Laboratories, Belmont, CA).[27,28] In addition, we also incubated cells in HG with either 10 ng/mL TGF-β1–neutralizing antibody (Sigma-Aldrich), 10 μM bosentan, or a combination of the two. Concentrations of peptides and inhibitors were in accordance with the manufacturer's recommendations. After a 24-hour incubation period, RNA was extracted for RT-PCR.

RNA Isolation and cDNA Synthesis

Total RNA from vitreous samples, retinal tissues, and endothelial cells was isolated (TRIzol; Invitrogen Inc., Burlington, Ontario, Canada) as previously described.[25–27] After RNA isolation, DNase treatment was performed to degrade any contaminating DNA in the samples (Invitrogen, Inc.). RNA was quantified by measuring ultraviolet absorbance at 260 nm. Purity of samples was assessed by determining optic density at 260:280 nm. cDNA was synthesized using 3 μg of total RNA with oligo-(dT) primers and Moloney murine leukemia virus (MMLV)-reverse transcriptase (Superscript II; Invitrogen Inc.).[25–27]

Real-Time RT-PCR

Real-time quantitative RT-PCR was performed in a real-time thermocycler (LightCycler; (Roche Diagnostics Canada, Montreal, Quebec, Canada) using the SYBR Green I detection platform (Molecular Probes, Eugene, OR). SYBR Green I is a double-stranded (ds)DNA–binding dye that allows kinetic quantification of PCR products. PCR reactions were performed in glass microcapillaries (Roche Diagnostics) in a total volume of 20 μL. The reaction mixture consisted of 2.5 μL of 10× PCR buffer (Invitrogen Inc.), 1.25 μL of 5 mM dNTP, 1.2 μL of 50 mM $MgCl_2$ (1.6 μL for rat TGF-β1 and ET-1), 1 μL of each forward and reverse 10 μM primer (Table 1),[28,29] 0.5 μL of 5 U/μL platinum DNA *Taq* polymerase, 0.75 μL of 10× SYBR Green I, 10.8 μL H_2O, and 1 μL cDNA template. For human ET-1 and TGF-β1, we designed minor groove-binding probes (*Taq*man; Applied Biosystems, Foster City, CA) on computer (Primer Express, ver. 2.0; Applied Biosystems) to avoid signal acquisition from nonspecific amplification products. These probes are modified at the 5′ end by the addition of 6-carboxyfluorescein (FAM) and at the 3′ end by the addition of a nonfluorescent quencher. As elongation proceeds, FAM is cleaved by the exonuclease activity of DNA *Taq* polymerase and an increase in reporter fluorescence emission takes place. The reporter dye (FAM, *Taq*man, Applied Biosystems) exhibits excitation and emission in the same range as SYBR I, which allows detection with the same detector channel. The PCR reaction mixture for human ET-1 and TGF-β1 was the same, with the exception of 0.75 μL of 15 μM probes instead of SYBR I.

During PCR reactions, an additional step was incorporated after the extension phase, to minimize signal interference from primer dimers and nonspecific amplification products.[30] This signal acquisition phase was empirically determined by obtaining the melting temperature (T_m) of all products by melting curve analysis (MCA). During MCA, post-PCR products were allowed to anneal at 60°C before the temperature was increased at 0.10°C per second. The signal acquisition step was set to 2°C to 3°C below the T_m of the specific amplification product (Table 2).

mRNA levels were quantified using the standard curve method. Standard curves for all genes were constructed by using serially diluted

TABLE 2. Temperature Profile for Real-Time RT-PCR

Gene	Species	Annealing (temperature-time)	Extension (temperature-time)	Signal Acquisition (temperature-time)
18S rRNA	Human/Rat	55°C-5 s	72°C-9 s	80°C-1 s
EDB+ FN	Human/Rat	55°C-5 s	72°C-8 s	81°C-1 s
EDA+ FN	Human/Rat	55°C-5 s	72°C-8 s	82°C-1 s
Total FN	Human	50°C-6 s	72°C-10 s	81°C-1 s
	Rat	60°C-5 s	72°C-8 s	84°C-1 s
TGF-β1	Human	59°C-5 s	72°C-4 s	After extension
	Rat	55°C-5 s	72°C-12 s	85°C-1 s
ET-1	Human	55°C-5 s	72°C-4 s	After extension
	Rat	58°C-5 s	72°C-20 s	84°C-1 s

Initial denaturation was performed at 94°C for 1 minute. Ramp rate for all PCR phases was 20°C per second.

standard template. Crossing point (*C*p; log-linear phase) was measured for all samples and used to compute mRNA levels from the standard curves. The data were normalized to a housekeeping gene (18S rRNA) to account for differences in reverse transcription efficiencies and the amount of template in the reaction mixtures.

Western Blot Analysis

Total protein from endothelial cells was isolated by homogenizing cells in complete RIPA buffer (NaCl 0.877 g, deoxycholate 1 g, 1 M Tris-HCl (pH 7.5) 5 mL, Triton X-100 1 mL, and 10% sodium dodecyl sulfate 1 mL; volume adjusted to 100 mL using ddH_2O) and protease inhibitor. Total proteins were then quantified by using the bicinchoninic acid (BCA) protein assay kit (Endogen; Pierce Biotechnology, Rockford, IL). ET-1 and TGF-β1 protein levels were measured by Western blot analysis. Polyclonal rabbit anti-human ET-1 (1:1000; Peninsula Laboratory) and rabbit anti-human TGF-β1 antibody (1:1000; Promega Corp., Madison, WI) were used followed by a secondary antibody conjugated with horseradish peroxide (HRP; 1:10,000). A Western blot detection kit (ECL-PLUS; Amersham Pharmacia Biotech, Piscataway, NJ) was used for detection.

Oncofetal FN Gene Silencing

We used a novel small interfering (si)RNA-based technique to silence specifically the EDB^+ oncofetal FN expression in endothelial cells. This technique is based on introduction of small dsRNA species that act as a guide in the nuclease complex and target cognate mRNA species.[31] siRNAs were constructed to target the EDB of oncofetal FN using an siRNA construction kit (Silencer; Ambion Inc., Austin, TX). Four potential sites in the EDB of human oncofetal FN mRNA were identified by scanning the domain for amino acid (AA) dinucleotide sequences. These sites differ from each other only in GC content. After identification of target sequences, oligonucleotides were synthesized for in vitro transcription and siRNA generation (Table 3). The oligonucleotides were synthesized by addition of a dinucleotide AA sequence at the 5′ end and an eight-nucleotide 5′-CCTGTCTC-3′ leader sequence at the 3′ end. This leader sequence is complementary to the T7 promoter primer used for in vitro transcription (Ambion Inc.). After synthesis, siRNA concentration was determined by measuring absorbance at 260 nm.

Endothelial cells were transfected with EDB^+ oncofetal FN siRNAs (100 nM final concentration) using siRNA transfection reagent (1 μL reagent per 500 μL transfection volume; siPORT Lipid; Ambion Inc.). Successful oncofetal FN siRNA transfection was demonstrated by significantly reduced levels of oncofetal FN transcripts after 72 hours of treatment, as assessed by real time RT-PCR. All siRNA experiments included transfection of endothelial cells with siRNAs which have no sequence homology (negative transfection) with human genome (negative control siRNAs; Ambion Inc.).

Cell Proliferation Assay

Endothelial cell proliferative capacity was assessed by 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT; Sigma-Aldrich, St. Louis, MO) assay as previously described.[32] Briefly, trypsinized endothelial cell suspension was added to a 96-well plate, and cells were allowed to attach in the presence of serum for 2 hours. Cells were then transfected with EDB^+ oncofetal FN siRNAs or negative control siRNAs in serum-free medium. After 72 hours of treatment, 50 μL XTT reagent (prepared by adding 25 μL of 5 mM phenazine methosulfate to 5 mL of 1 mg/mL XTT) was added to the wells, and cells were incubated for 4 hours. Absorbance was measured at 450 nm to assess cell proliferation.

Statistical Analysis

The data are expressed as the mean ± SEM and were analyzed by ANOVA followed by Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Upregulation of Oncofetal FN, TGF-β1, and ET-1 in Vitreous Samples from Patients with PDR

We used a novel and sensitive real-time RT-PCR method to quantify expression of oncofetal FN isoforms in vitreous sam-

TABLE 3. Oligonucleotide Sequences for In Vitro Transcription and siRNA Synthesis

	siRNA Sequences* (5′→3′)	Nucleotides†
EDB1 SiRNA	Oligo 1 AAGGTATCCCTATTTTTGAAGCCTGTCTC	1423–1441
	Oligo 2 AACTTCAAAAATAGGGATACCCCTGTCTC	
EDB2 SiRNA	Oligo 1 AACTCACTGACCTAAGCTTTGCCTGTCTC	1414–1432
	Oligo 2 AACAAAGCTTAGGTCAGTGAGCCTGTCTC	
EDB3 SiRNA	Oligo 1 AACCGATTCAAGCATCGGCCTCCTGTCTC	1386–1404
	Oligo 2 AAAGGCCGATGCTTGAATCGGCCTGTCTC	
EDB4 SiRNA	Oligo 1 AAGCATCGGCCTGAGGTGGACCCTGTCTC	1500–1518
	Oligo 2 AAGTCCACCTCAGGCCGATGCCCTGTCTC	

* Sequence targeted is shown in bold.

† Nucleotide position in reference to Human EDB^+ FN sequence (accession number, X07717).

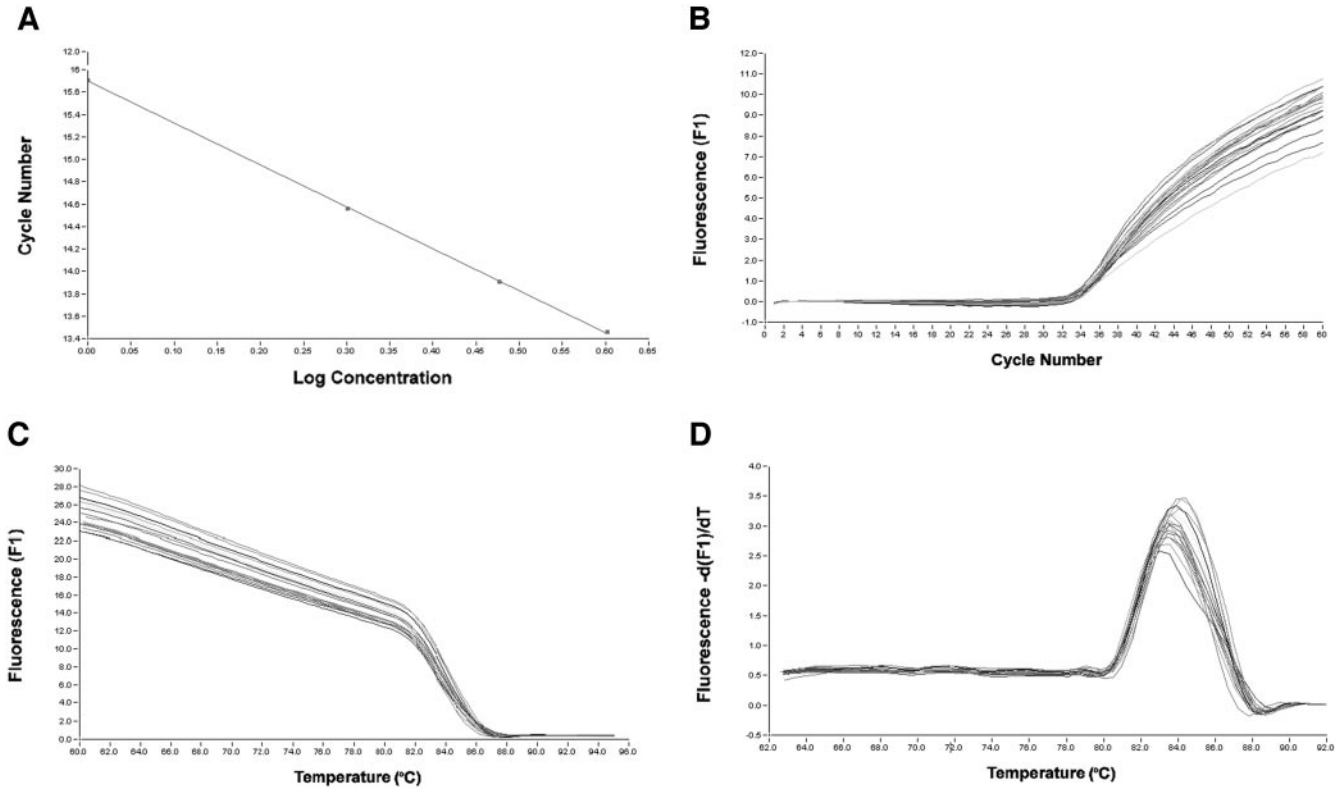

FIGURE 1. Real-time RT-PCR of EDB$^+$ FN in human vitreous samples (each sample shown by a different trace). Real-time RT-PCR amplification of human EDB$^+$ FN from vitreous samples, showing (**A**) standard curve constructed for human EDB$^+$ FN by serial dilution of standard template, (**B**) PCR amplification plot of EDB$^+$ FN from human vitreous samples, (**C**) melting curve analysis of PCR products in (**B**), and (**D**) melting peak data (derivative plot) denoting a single specific dsDNA species in the reaction mixture. Real time RT-PCR allows for kinetic measurement of PCR products in log-linear phase. *C*p (*x*-axis; start of log-linear phase) was used to compute the relative levels of target mRNA from the standard curve. All PCR reactions were coupled to melting-curve analysis to determine specificity of amplification.

ples from patients with PDR (Fig. 1). Our findings indicate that diabetes increased the level of total FN expression in the vitreous of patients with PDR compared with control subjects ($P < 0.01$; Fig. 2A), along with upregulation of EDB$^+$ FN ($P < 0.01$; Fig. 2B). However, there was no difference in the expression of EDA$^+$ FN among diabetic and nondiabetic patients (Fig. 2C). Our data further indicate co-upregulation of TGF-β1 and ET-1 in vitreous of diabetic subjects ($P < 0.01$; Figs. 2D, 2E). It is interesting to note that the level of expression of oncofetal FN in nondiabetic control subjects paralleled the presence of a fibrotic epiretinal membrane (data not shown). Our data indicate that, in addition to direct upregulation of FN, there is a preferential expression of the fetal form of FN in the vitreous of diabetic subjects. Furthermore, concurrent upregulation of TGF-β1 and ET-1 suggests a potential role of these factors in mediating differential expression of FN.

Diabetes-Induced Upregulation of Oncofetal FN, TGF-β1, and ET-1 in Rats

We used streptozotocin (STZ)-induced diabetic rats, a model of chronic diabetes, after a 3-month follow-up to examine retinal FN isoform expression and the possible involvement of TGF-β1 and ET-1. Diabetes was confirmed by blood glucose estimation, body weight gain, and glycated hemoglobin levels in rats. Diabetic animals exhibited hyperglycemia compared with normoglycemic control subjects (Table 4). In addition, diabetic animals demonstrated reduced body weight gain and elevated glycated hemoglobin levels. These parameters indicate diabetic dysmetabolism in these animals. We also treated the diabetic rats with the dual ET receptor antagonist bosentan. There was no significant difference between diabetic nontreated animals and diabetic animals treated with bosentan in any of the parameters used for clinical monitoring. In addition, systolic blood pressure, recorded by tail plethysmography, did not differ among the animal groups (Table 4).

Our results of retinal mRNA confirm previous findings that diabetes leads to upregulation of FN expression in the retina ($P < 0.05$; Fig. 3A). In addition, diabetic rats showed increased expression of EDB$^+$ FN and EDA$^+$ FN ($P < 0.05$; Figs. 3B, 3C). Treatment of diabetic rats with bosentan prevented upregulation of both total FN and EDB$^+$ FN but exerted, although significant, a somewhat less pronounced effect on EDA$^+$ FN expression. TGF-β1 and ET-1 mRNA levels were also increased in the retinas of diabetic animals ($P < 0.05$; Fig. 3D, 3E). Bosentan treatment significantly reduced mRNA levels of both TGF-β1 and ET-1. This suggests a possible regulation of TGF-β1 expression through ET-receptor–mediated signaling in diabetes. Our data further indicate that hyperglycemia-induced upregulation of TGF-β1 and ET-1 and their possible interaction may be involved in differential FN expression in retinas of diabetic rats.

Glucose-Induced Expression of Oncofetal FN in Endothelial Cells and Its Regulation by TGF-β1 and ET-1

The major targets of sustained hyperglycemia in retinal vasculature are endothelial cells. These cells are the first to encounter HG in the circulation and feature early biosynthetic changes that are possibly integral to the pathogenesis of diabetic retinopathy.[33,34] We chose endothelial cells to elucidate the biochemical and molecular pathways leading to aberrant expression of oncofetal FN because endothelial cells have the capability of producing EDB$^+$ FN,[35] and because these cells are

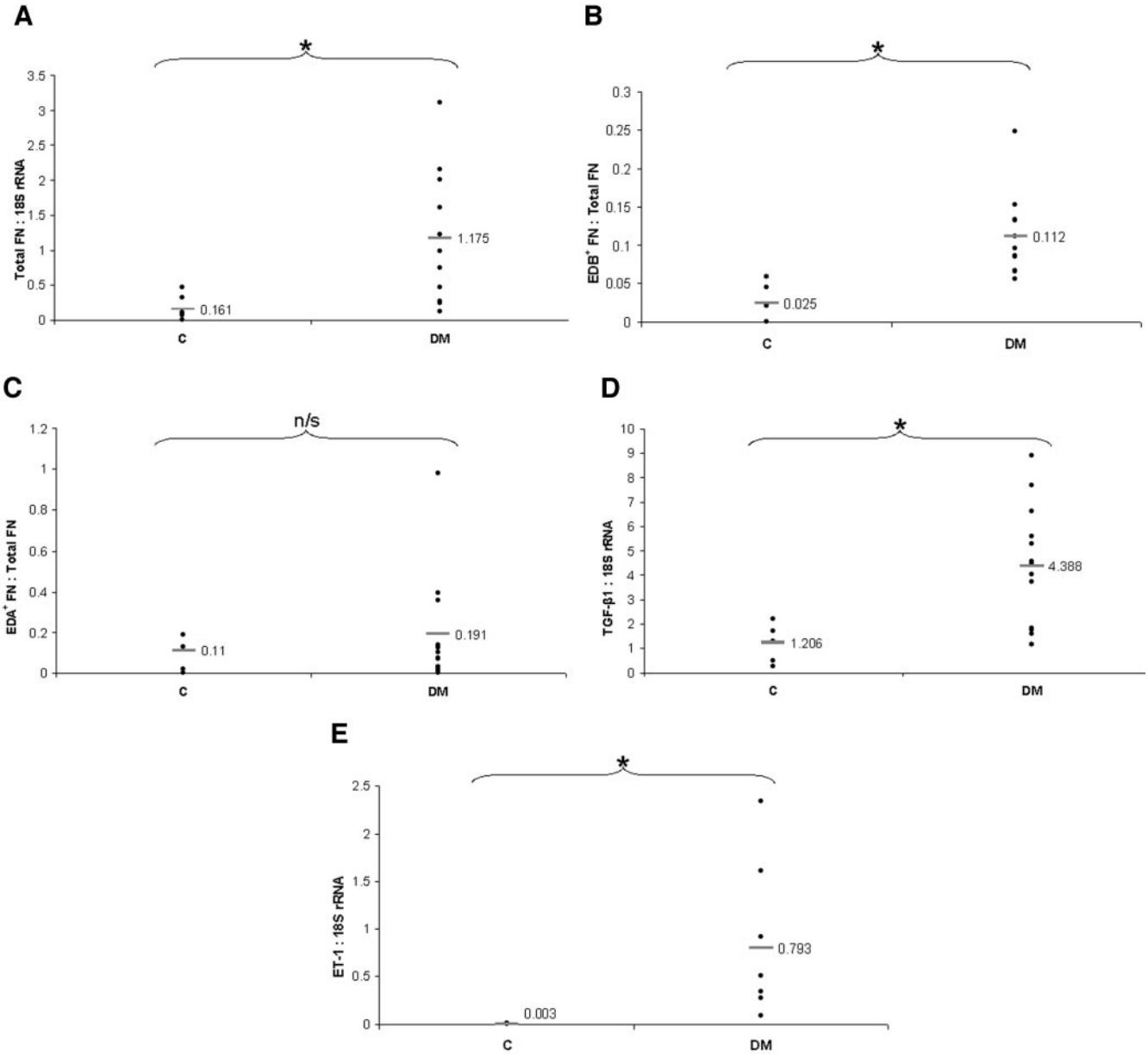

FIGURE 2. Expression of FN isoforms in human vitreous. Quantification of FN isoform expression in vitreous samples by real-time RT-PCR, showing (**A**) total FN, (**B**) EDB^+ FN, (**C**) EDA^+ FN, (**D**) TGF-β1, and (**E**) ET-1 mRNA levels. Total FN, TGF-β1, and ET-1 mRNA levels are expressed as the ratio of target mRNA to 18S rRNA. FN isoform levels are expressed as the ratio of isoform mRNA to total FN mRNA. C, nondiabetic group (n = 6); DM, diabetic group (n = 18). *Horizontal line*: mean of the respective group. (*$P < 0.01$ compared with C; n/s, no significant difference from C.)

fundamental in several stages of the process of angiogenesis. In addition, FN has been shown to be predominantly present at the basal and luminal surfaces of endothelial cells in intraocular vascularized tissue of patients with PDR.[36]

Microvascular endothelial cells were incubated in either normal glucose or HG. Cells in normal glucose were also stimulated with TGF-β1 or ET-1 peptide. In addition, cells incubated in HG were treated with neutralizing TGF-β1 anti-

TABLE 4. Clinical Monitoring of Animals

	Group		
Biophysical Parameters	**C**	**DM**	**DM-B**
Blood glucose (mg/dL)	77.4 ± 1.7	*412.4 ± 4.1	*423.6 ± 5.9
Glycated hemoglobin (%)	5.2 ± 0.7	*18.2 ± 0.9	*17.8 ± 0.5
Body weight (g)	632.0 ± 26.7	*473.0 ± 17.2	*481.0 ± 18.4
Systolic blood pressure (mm Hg)	104.0 ± 7.0	110.0 ± 4.0	107.0 ± 9

* $P < 0.05$ as compared to controls (C).

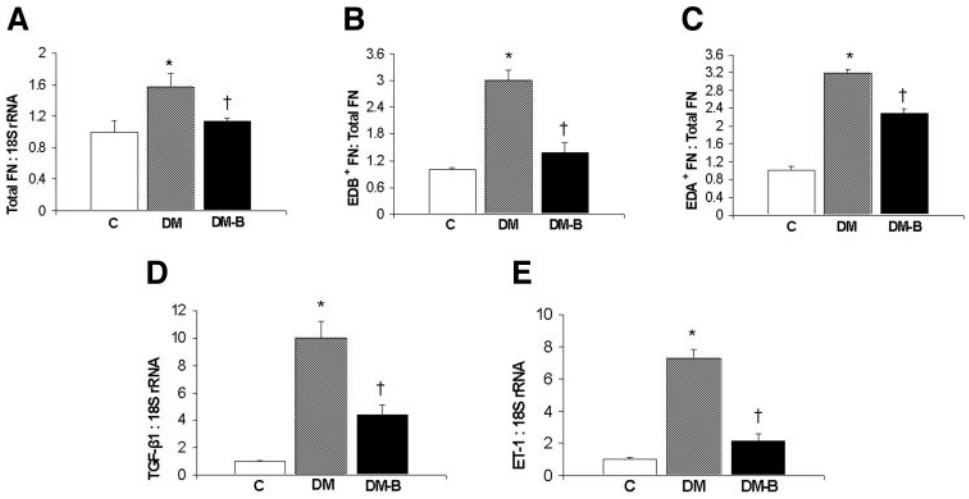

FIGURE 3. Upregulation of oncofetal FN in retinas of diabetic rats. Quantification of FN isoform expression in retinas of SD rats by real-time RT-PCR, showing (**A**) total FN, (**B**) EDB^+ FN, (**C**) EDA^+ FN, (**D**) TGF-β1, and (**E**) ET-1 mRNA levels. Total FN, TGF-β1 and ET-1 mRNA levels are expressed as ratio of target mRNA to 18S rRNA (relative to control). FN isoform levels are expressed as ratio of isoform mRNA to total FN mRNA (relative to control). C, non-diabetic group ($n = 6$); DM, poorly controlled diabetic group ($n = 6$); and DM-B, poorly controlled diabetic group with bosentan treatment ($n = 6$). Data presented as mean ± SEM. (*$P < 0.05$ compared with C; †$P < 0.05$ compared with DM-B.)

body, bosentan, or a combination of both. Our data indicate that a high glucose concentration led to upregulation of total FN in endothelial cells ($P < 0.05$; Fig. 4A). The relative levels of EDB^+ FN and EDA^+ FN mRNA were also increased by HG ($P < 0.05$; Figs. 4B, 4C). In addition, mRNA levels of TGF-β1 and ET-1 were upregulated in cells exposed to a high glucose concentration ($P < 0.05$; Figs. 4D, 4E). The mediators involved in hyperglycemia-induced upregulation of total FN, EDB^+ FN, and EDA^+ FN seem to be TGF-β1 and ET-1, as incubation of cells with these peptides produced results similar to those with HG. This role of hyperglycemia-induced TGF-β1 and ET-1 was further confirmed with the observation that cells incubated in HG with either neutralizing TGF-β1 antibody or bosentan normalized the increase in total FN and in the oncofetal isoforms. In addition, incubation of cells exposed to HG with TGF-β1–neutralizing antibody and bosentan completely prevented glucose-induced upregulation of total FN, oncofetal FN, TGF-β1, and ET-1 transcript levels. It is also evident that there was a considerable cross-interaction between TGF-β1 and ET-1 by which the two peptides modulated the expression of each other. Incubation of endothelial cells exposed to a high glucose concentration with TGF-β1–neutralizing antibody and bosentan led to downregulation of both ET-1 and TGF-β1 mRNA levels, respectively.

To elucidate whether alteration of mRNA levels coincides with alteration of protein levels, we performed Western blot analysis of both potential regulators of oncofetal FN expression, TGF-β1 and ET-1. Our data indicate upregulation of TGF-β1 and ET-1 protein levels by HG ($P < 0.05$; Fig. 5). In parallel to our mRNA data, TGF-β1 and ET-1 protein levels were found to be increased in cells exposed to 5 mM glucose and either TGF-β1 peptide or ET-1 peptide. Similarly, neutralization of TGF-β1 activity and inhibition of ET-receptor–mediated signaling prevented HG-induced upregulation of both TGF-β1 and ET-1 protein expression.

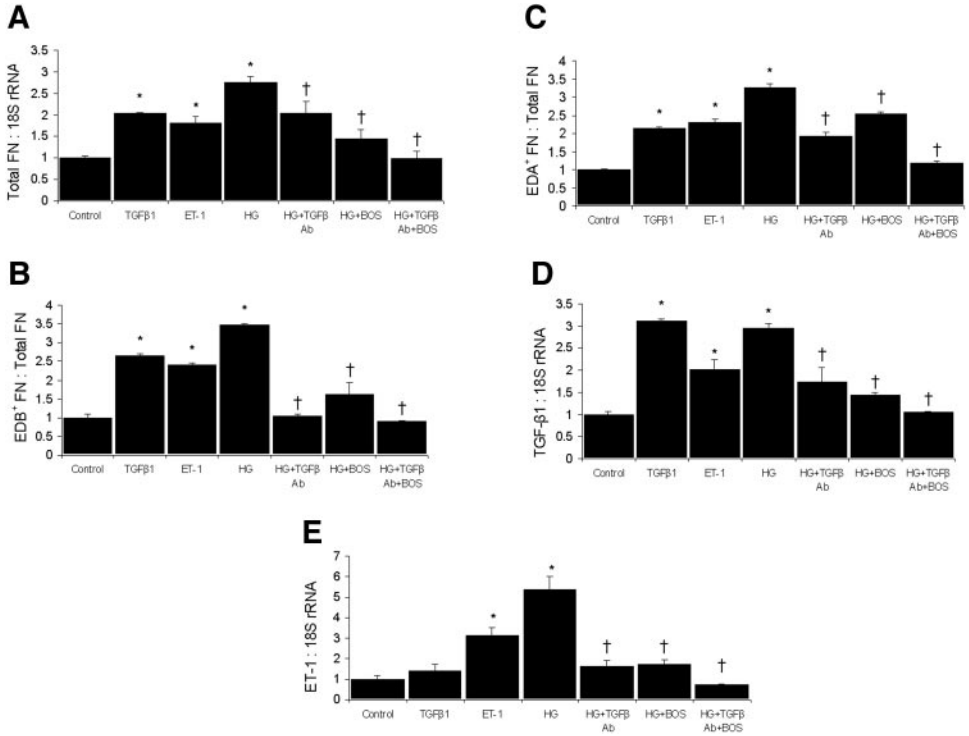

FIGURE 4. Regulation of oncofetal FN through hyperglycemia-induced TGF-β1 and ET-1 in endothelial cells. Effect of high glucose concentration, TGF-β1, and ET-1 peptide on endothelial FN isoform expression as determined by real-time RT-PCR, showing (**A**) total FN, (**B**) EDB^+ FN, (**C**) EDA^+ FN, (**D**) TGF-β1, and (**E**) ET-1 mRNA levels. Total FN, TGF-β1, and ET-1 mRNA levels are expressed as the ratio of target mRNA to 18S rRNA (relative to control). FN isoform levels are expressed as the ratio of isoform mRNA to total FN mRNA (relative to control). Control, normal (5 mM) glucose; TGFβ1, normal glucose+TGF-β1; ET-1, normal glucose +ET-1; HG, high (25 mM) glucose; HG+TGFβ Ab, high glucose+TGF-β1–neutralizing antibody; HG+BOS, high glucose+bosentan; HG+TGFβAb+BOS, high glucose+TGF-β1–neutralizing antibody+bosentan. Data are expressed as the mean ± SEM ($n = 5$ per treatment). ($P < 0.05$ compared with control; †$P < 0.05$ compared with HG.)

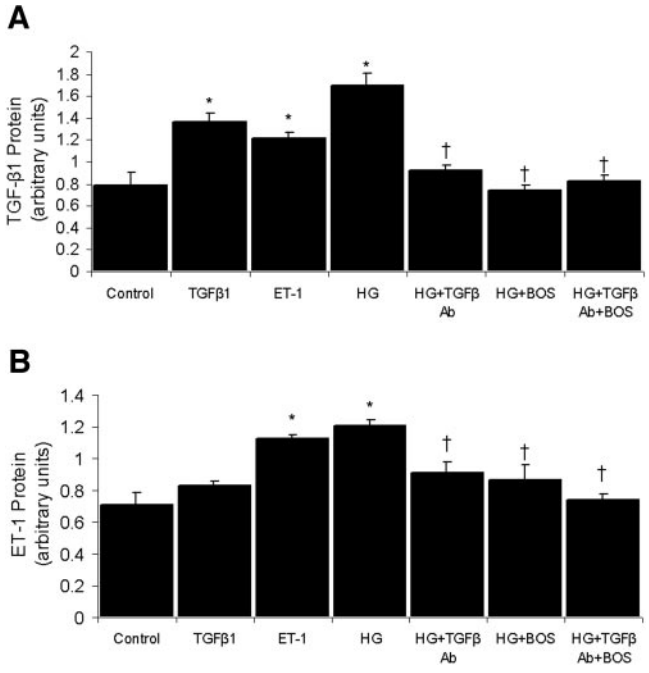

FIGURE 5. TGF-β1 and ET-1 protein expression in endothelial cells. Quantification by Western blot analysis of (**A**) TGF-β1 and (**B**) ET-1 protein expression in endothelial cells exposed high glucose concentration, TGF-β1 peptide, and ET-1 peptide. Abbreviations, expression of data, and probabilities are as in Figure 4.

Oncofetal FN in Endothelial Cell Proliferation

EDB^+ oncofetal FN has been proposed to be a marker of tumor angiogenesis.[16–19] Upregulation of EDB^+ oncofetal FN during angiogenesis suggests a key role in vascular endothelial cell proliferation. To determine the functional significance of diabetes-induced EDB^+ FN upregulation, we performed an in vitro proliferation assay after inhibition of oncofetal FN expression in endothelial cells. Transfection of endothelial cells with siRNAs targeted to EDB resulted in significantly lower transcript levels of EDB^+ FN compared with negative control transfected cells ($P < 0.05$; Fig. 6A). Furthermore, inhibition of EDB^+ oncofetal FN expression reduced the proliferative capacity of endothelial cells compared with negative control transfected cells, as measured by an XTT assay ($P < 0.05$; Fig. 6B).

DISCUSSION

In the present study, we demonstrated for the first time the alteration of oncofetal FN in diabetic retinopathy. Both in animal experiments, relatively early in the disease course, and in human vitreous, denoting advanced diabetic retinopathy, expression of oncofetal FN isoform was upregulated. The mechanistic basis for the regulation of oncofetal FN was elucidated in endothelial cells, the major cell types involved in FN expression in the retinal vasculature. We demonstrated that hyperglycemia-induced TGF-β1 and ET-1 are not only involved in direct upregulation of total FN but also are mediators of preferential expression of embryonic isoforms of FN. In addition, our results indicate an important role for oncofetal FN in endothelial cell proliferation.

The inclusion of the oncofetal domains in the FN molecule is regulated in an oncodevelopmental manner. Oncofetal FN has been proposed to act as a key molecule in tumoral angiogenesis.[16–19] In support of this suggestion is the observation that endothelial cells cultured with recombinant EDB^+ FN show a higher proliferative index.[37] Despite accumulated evidence of regulated spatial and temporal expression of oncofetal FN, the biological significance remains to be determined. Recently, FN molecules containing EDB and EDA were shown to promote cell adhesion and migration, respectively.[38,39] It has been suggested that insertion of these oncofetal domains causes a global conformational change in the FN molecule improving access to integrin-binding sites.[40]

The exact role of oncofetal FN in diabetic retinopathy is obscure. However, based on the data in nondiabetic situation, one may speculate that oncofetal FN is a key molecule in the production of structural changes in diabetic retinopathy such as CBMT and angiogenesis. The presence of oncofetal FN in human vitreous, as demonstrated in the present study, supports this viewpoint. Our results suggest that oncofetal FN molecules are of importance in endothelial cell proliferation and may be involved in angiogenesis. It is possible that these embryonic forms of FN are reexpressed to provide FN species that are potentially more appropriate for endothelial cell migration and proliferation leading to angiogenesis. Whether presence of oncofetal FN may serve as a diagnostic marker for retinal neovascularization or as a therapeutic target remains to be investigated.

Various growth factors and cytokines have been implicated in the pathogenic interactions that cause progression of retinopathy to advanced stages.[41,42] Of these numerous factors, ETs and TGF-β have gained significant attention because of their direct effects on ECM protein synthesis.[6,23,43,44] We have demonstrated that ET-1 and -3 immunoreactivity and mRNA levels are increased in the retinas of diabetic animals and that ET-receptor antagonism prevents CBMT in diabetes.[6,45,46] In contrast, TGF-β has long been accepted as a key regulator of increased ECM protein synthesis in diabetic nephropathy and a number of other fibrotic diseases.[23,44] There is accumulating

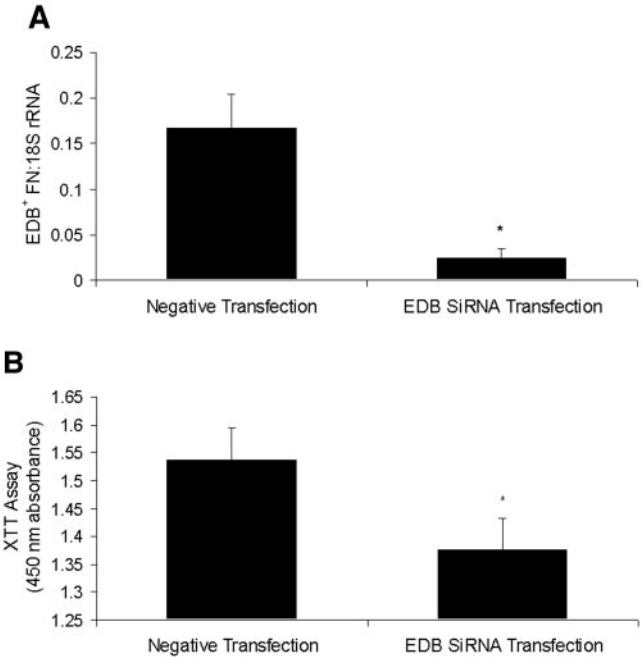

FIGURE 6. Oncofetal FN–mediated endothelial cell proliferation. Effect of EDB-targeting siRNAs, showing (**A**) endothelial EDB^+ oncofetal FN expression, as determined by real-time RT-PCR and (**B**) endothelial proliferation as determined by XTT assay. Oncofetal FN levels are expressed as the ratio of isoform mRNA to 18S rRNA. Endothelial cell proliferation was assessed by measuring absorbance at 450 nm after incubation with XTT reagent. Negative transfection, negative control; EDB siRNA transfection, siRNAs targeted to EDB of oncofetal FN. Data expressed as the mean ± SEM ($n = 5$ per treatment). (*$P < 0.05$ compared with negative transfection.)

evidence that these two growth factors may also regulate each other.[47,48] Our findings point toward an interactive role between these two fibrogenic proteins. A common denominator in this regulatory interaction may be PKC activation[3,4,49,50]; therefore, one of these factors leads to activation of PKC and subsequent upregulation of the other. This interactive relationship between infamous fibrogenic proteins could be responsible for regulating composition of the ECM. We have shown that increased expression of oncofetal FN in vitreous samples from patients with PDR and retinal tissues from diabetic rats exhibited concurrent upregulation of TGF-β1 and ET-1. Furthermore, incubation of endothelial cells with TGF-β1 or ET-1 leads to preferential expression of EDA^+ FN and EDB^+ FN. It should be noted that other angiogenic factors, such as vascular endothelial growth factor, may also regulate the expression of oncofetal FN, an avenue that remains to be investigated.

Although no previous study has investigated EDB^+ FN in diabetic retinopathy, EDA^+ FN in retinal microvessels of diabetic patients has been examined with no conclusive evidence of any alteration.[7] In keeping with the findings of the aforementioned study, we have demonstrated that there is no significant difference in EDA^+ FN mRNA levels in the vitreous of diabetic and nondiabetic patients. However, retinal tissues from STZ-induced diabetic rats and endothelial cells exposed to a high glucose concentration show an upregulation of EDA^+ FN mRNA levels. It is plausible that increased EDA^+ FN expression is an early event in diabetes-induced retinal dysfunction. Data obtained from retinas of diabetic rats in the early stage of retinopathy, and from human vitreous samples in the advanced stage of retinopathy, certainly support the idea of EDA^+ FN expression as an early event. EDB^+ FN, however, was upregulated in both the vitreous of diabetic patients and retinal tissues of diabetic rats. It is possible that EDB^+ FN exhibits a sustained elevated level of expression during the course of diabetes-induced pathogenic changes in the retina.

In conclusion, our data showed that diabetes-induced upregulation of oncofetal FN may be mediated by TGF-β1 and ET-1 and is a novel mechanism that may be involved in the pathogenesis of diabetic retinopathy. This may have great potential as a diagnostic marker of diabetic retinopathy and, similar to cancer therapy may act as a therapeutic target.

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