Retina

Regulation of Vascular Endothelial Growth Factor Expression by Extra Domain B Segment of Fibronectin in Endothelial Cells

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Purpose. Diabetic retinopathy entails proliferation of vascular endothelial cells (ECs) and unregulated angiogenesis. We have previously shown that ECs increase the expression of an embryonic variant of fibronectin (FN), called extra domain-B FN (ED-B FN) in response to high glucose. We also showed that ED-B FN regulates EC tube morphogenesis, possibly through vascular endothelial growth factor (VEGF). In the present study, we have attempted to decipher the mechanisms by which ED-B FN may modulate EC phenotype.

Methods. We hypothesized that ED-B FN regulates VEGF expression in ECs through interaction with selected integrin receptors. To test this hypothesis, we first cultured ECs in high levels of glucose to investigate for any alteration. We then used integrin-specific matrix mimetic peptides, neutralizing antibodies, and RNAi to identify the integrin(s) involved in VEGF expression. Finally, we used an animal model of diabetes to study whether these in vitro mechanisms also take place in the retina.

Results. Our results show that exposure of ECs to high levels of glucose increased VEGF expression. ED-B FN mediated this increase since knockdown of ED-B FN completely prevented glucose-induced VEGF expression. We then identified β1 integrin as the essential receptor involved in high glucose-induced VEGF expression. We also showed that diabetes increased β1 integrin and VEGF expression in the retina, which normalized upon ED-B knockdown.

Conclusions. These findings showed that high levels of glucose in diabetes increased VEGF expression in ECs through ED-B FN and β1 integrin interaction. These results provide novel mechanistic basis of increased VEGF expression in diabetes. (*Invest Ophthalmol Vis Sci.* 2012;53:8333–8343) DOI: 10.1167/iovs.12-9766

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Diabetic retinopathy (DR), a microvascular complication of longstanding diabetes, is the most common cause of blindness in the working population. All people with diabetes (types 1 and 2) eventually develop some form of retinopathy.[1] Early pathogenic events in DR include altered expression of vasoactive factors and basement membrane (BM) proteins. Ultimately, endothelial cell (EC) dysfunction ensues. The loss of vasoregulation, thickening of the BM, increased permeability, and the eventual EC loss converge to create an ischemic retina.[2,3] In response, new blood vessels form, however, this reactive angiogenesis is unregulated and leads to blindness.[2–4]

The mechanisms of unregulated reactive angiogenesis in DR are becoming known. We know that a number of growth factors are elaborated, including the EC-specific mitogen, vascular endothelial growth factor (VEGF).[2] Numerous VEGF isoforms arise from alternative splicing.[5–7] These include isoforms of 121, 145, 148, 165, 183, 189, and 206 amino acids.[8] VEGF121 is the most soluble form, whereas VEGF189 and VEGF206 are almost exclusively sequestered in the extracellular matrix (ECM). VEGF binds to VEGF receptors (VEGFR1 and VEGFR2) to alter EC properties such as proliferation and permeability.[9–11] Evidence for a role of VEGF in DR comes from studies that show elevated levels in the vitreous samples of patients with active late-stage DR.[12–14] In experimental models, chimeric VEGF receptor proteins (which sequester VEGF) have been shown to suppress neovascularization in almost all animal models studied.[15–17]

The source of elevated VEGF in DR is not fully elucidated, but may include retinal ECs and pigment epithelial cells.[2,18,19] EC-derived VEGF has been recently shown to be an essential autocrine mechanism for cell survival.[20,21] We and others have shown that culture of ECs in high levels of glucose increases VEGF mRNA and protein levels.[22,23] The signaling pathway involved in high glucose–induced VEGF expression remains enigmatic. One possible mechanism may be through altered ECM. ECM of cells expressing VEGF can stimulate proliferation.[24] This could be because of sequestration of VEGF in the ECM or because the ECM composition of VEGF-expressing cells is different. We have demonstrated that high levels of glucose, which increase VEGF expression, also alter the composition of EC-derived ECM proteins. Specifically, we reported increased expression of an embryonic splice variant of fibronectin (FN) (called extra domain-B FN [ED-B FN]) by ECs challenged with high levels of glucose. Targeted inhibition of ED-B FN caused decreased EC proliferation and tube morphogenesis.[22] These two cellular activities are reminiscent of EC response to VEGF. Therefore, we hypothesized that ED-B FN regulated autogenous VEGF expression in ECs. This ECM protein-mediated VEGF expression may be due to an interaction between matrix protein receptors on EC surface. In the present study, we tested this hypothesis in cultured ECs. Using integrin-binding peptides, neutralizing antibodies, and RNAi, we showed that ED-B caused

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TABLE. Primer Sequences for RT-PCR

Genes (sp)	Sequence (5′ → 3′)
β1 integrin (hu)	CGAGGTCATGGTTCATGTTC
	CAGTGTTGTGGGATTTGCAC
β1 integrin (mo)	GGTGTCGTGTTTGTGAATGC
	CTCCTGTGCACACGTGTCTT
ED-B FN (hu/mo)	CCGCCATTAATGAGAGTGAT
	AGTTAGTTGCGGCAGGAGAAG
Total FN (hu)	GATAAATCAACAGTGGGAGC
	CCCAGATCATGGAGTCTTTA
VEGF (hu)	ATCTTCAAGCCATCCTGTGTGC
	GCTCACCGCCTCGGCTTGT
VEGF (mo)	CCATGAACTTTCTGCTCTCTTG
	GGTGAGAGGTCTGGTTCCCGAA
VEGF121 (hu)	ATCTTCAAGCCATCCTGTGTGC
	TGCGCTTGTCACATTTTTCTTG
VEGF145 (hu)	ATCTTCAAGCCATCCTGTGTGC
	TCGGCTTGTCACATACGCTCC
VEGF148 (hu)	ATCTTCAAGCCATCCTGTGTGC
	TCGGCTTGTCACATCTTGCAAC
VEGF165 (hu)	ATCTTCAAGCCATCCTGTGTGC
	CAAGGCCCACAGGGATTTTC
VEGF183 (hu)	ATCTTCAAGCCATCCTGTGTGC
	GCCCACAGGGACGGGATTT
VEGF189 (hu)	ATCTTCAAGCCATCCTGTGTGC
	CACAGGGAACGCTCCAGGAC
VEGFR1 (hu)	AGGGGAAGAAATCCTCCAGA
	CGTGCTGCTTCCTGGTCC
VEGFR2 (hu)	GTGACCAACATGGAGTCGTG
	TGCTTCACAGAAGACCATGC
β-actin (hu/mo)	CCTCTATGCCAACACAGTGC
	CATCGTACTCCTGCTTGCTG
18S rRNA (hu/mo)	GTAACCCGTTGAACCCCATT
	CCATCCAACGGTAGTAGCG

Hu, human; mo, mouse.

increased VEGF expression through interaction with β1 integrin.

METHODS

EC Culture

Human umbilical vein endothelial cells (HUVECs) (Cat # PCS-100-010; American Type Culture Collection, Rockville, MD) were plated at 2500 cells/cm^2 in endothelial basal medium (EBM) (CC-3121; Lonza Inc., Walkerville, MD) with endothelial growth supplements (EGM Single-Quots, CC-4133; Lonza Inc.) and 2% fetal bovine serum. Human retinal microvascular endothelial cells (HRMECs) (Cat # HEC09; Olaf Pharmaceuticals, Worcester, MA) were cultured in EBM2 media (CC-3156; Lonza Inc.) supplemented with 20% fetal bovine serum and endothelial growth supplements (EGM-2 SingleQuots, CC-4176; Lonza Inc.). Before performing experiments, cells were washed in PBS and incubated in basal media containing 1% FBS for 24 hours. To study the effect of high glucose levels, we cultured ECs in 25 mmol/L D-glucose (with 1% FBS). L-glucose (25 mmol/L) was used as the osmotic control. All experiments were carried out after 24 hours of glucose incubation unless otherwise indicated. At least three different batches of cells, each in triplicate, were used for each experiment.

Cellular Activity Assays

Tube formation assay was performed as described by us previously.[22] Briefly, we plated ECs on growth factor–reduced matrix (Matrigel; BD Biosciences, Mississauga, Ontario, Canada). After allowing cells to attach, we washed the plates with PBS and overlaid the cells with a collagen gel.[25] Type 1 collagen (C7661; Sigma-Aldrich, Oakville, Ontario, Canada) was prepared by mixing seven parts of a 1.4 mg/mL type I collagen with one part EBM2 basal media and with two parts of an 11.8 mg/mL sodium bicarbonate. Two investigators assessed EC branching after 24-hour treatment.

Cell viability and proliferation were determined by 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1; Roche, Laval, Quebec, Canada) assay. ECs were seeded onto 96-well plates at a density of 1.0×10^4 cells per well in 100 μL of culture medium with or without specific reagents for 24 hours. To measure the number of viable cells, 10 microliters of WST-1 was added per well, and the cells were incubated for 4 hours at 37°C. We then measured absorbance at 450 nm.

Integrin-Binding Peptides, ED-B FN Peptide, and Neutralizing Antibodies

ED-B FN peptide (sequence as reported in Khan et al.[22]) was obtained from Sigma-Aldrich and used at 2 μg/mL concentration. We have previously shown that 2 μg/mL ED-B peptide increases EC tube formation and enhances EC proliferation.[22] To examine the effect of VEGF on FN expression, we treated ECs with 10 ng/mL VEGF (VEGF165) (293-VE-010; R & D Systems, Minneapolis, MN) for 24 hours (HUVECs and HRMECs) or 48 hours (HRMECs). Integrin-binding peptides were used at 5 mmol/L concentration. The peptides included DGEA (α2β1 binding) (H-Asp-Gly-Glu-Ala-OH; Bachem Americas Inc., Torrance, CA), KDGE (α2β1 scramble sequence) (H-Lys-Asp-Gly-Glu-OH; Bachem Americas Inc.), and RGDS (α5β1) (H-Arg-Gly-Asp-Ser-OH; Bachem Americas Inc.).[26] Integrin α2β1, α5β1, β1, and β3 antibodies were obtained from Millipore (Temecula, CA) and used at 0.1 to 0.5 μg/mL. ECs were pretreated with the integrin-binding peptides or the neutralizing antibodies for 30 minutes before exposure to high glucose or ED-B peptide. For some experiments, we coated the culture plates with ED-B peptide (1 μg/cm^2) before treating the cells with IgG control antibody or integrin neutralizing antibodies.

siRNA Transfections

We used small interfering RNA (siRNA) to silence the expression of ED-B FN and integrins in ECs. Four pooled ED-B FN siRNAs (100 nmol/L final concentration) were used in a single reaction using lipid siRNA transfection reagent (1 μL reagent per 500 μL transfection volume) (siPORT; Ambion Inc., Burlington, Ontario, Canada). The sequence of the siRNAs is the same as shown by us previously.[22] Predesigned and validated integrin β1 (ITGB1) and β3 (ITGB3) siRNA were obtained from (gene IDs: 3688, 3690, respectively; Dharmacon Inc., Chicago, IL). Scrambled siRNAs were used as controls. siRNA transfection efficiency was assessed by measuring mRNA expression by real time reverse transcriptase-PCR (RT-PCR). We performed all experiments at optimal conditions with at least 75% knockdown.

Animal Model of Chronic Diabetes

Male B6 mice (Charles River Canada Ltd., Quebec, Canada), weighing approximately 20 g, were randomly divided into four groups ($n = 6$ per group): nondiabetic controls (Control), nondiabetic animals treated with ED-B siRNA (ED-B siRNA), diabetic animals treated with scrambled siRNA (Diab), and diabetic animals treated with ED-B siRNA (Diab + ED-B siRNA). Diabetes was induced by three intravenous injections of streptozotocin (STZ) (65 mg/kg in citrate buffer), while the control animals received the same volume of citrate buffer. Animals received 150 μg ED-B siRNAs weekly for 4 weeks by intravenous route (tail vein) using Lipofectamine 2000 transfection reagent. The animals were sacrificed 1 week after the fourth injection, and the retinal tissues were collected for gene expression analyses. All animal were treated in accordance with the ARVO Statement for the Use of Animals in

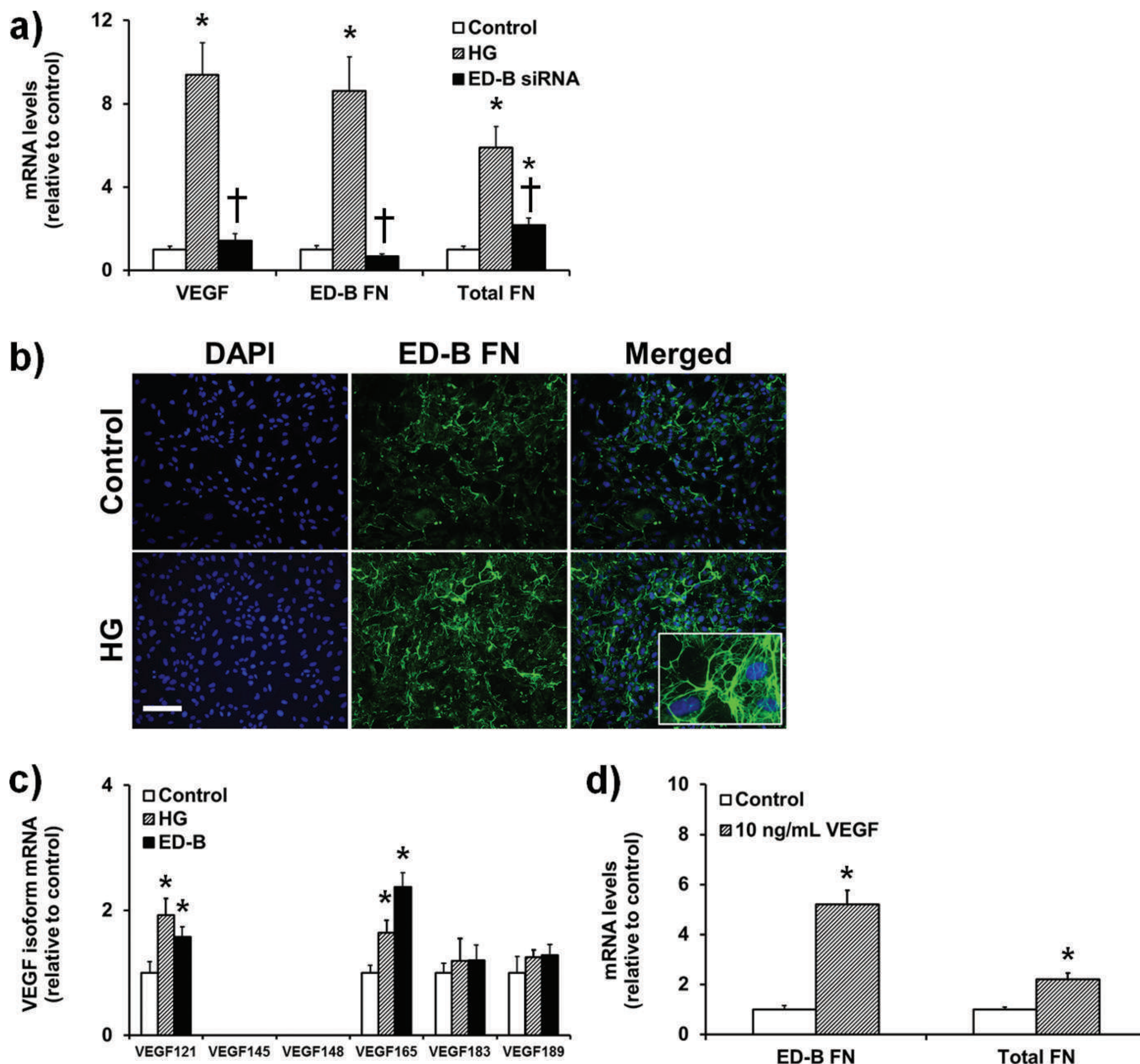

FIGURE 1. Expression of ED-B FN and VEGF in HUVECs. (**a**) Exposure of HUVECs to 25 mmol/L glucose (HG) caused increased mRNA expression of VEGF, ED-B FN, and total FN compared to 5 mmol/L glucose (Control) after 24 hours. This glucose-induced expression of VEGF and ED-B FN was prevented when HUVECs were transfected with ED-B FN siRNA. **P* < 0.05 compared to control, †*P* < 0.05, compared to HG. (**b**) Immunofluorescence staining for ED-B FN in HUVECs exposed to HG for 24 hrs (images taken at 20x; *scale bar* = 100 μm; insert showing high power image; *green* = ED-B FN, exposure time 16 seconds, and *blue* = DAPI, exposure time 3 seconds). (**c**) RT-PCR analysis of VEGF isoform expression in HUVECs exposed to HG or 2 μg/mL ED-B peptide. **P* < 0.05 compared to control. (**d**) Expression of FN in cells treated with 10 ng/mL recombinant VEGF165 for 24 hours. **P* < 0.05 compared to control.

Ophthalmic and Vision Research. All experiments were approved by the University of Western Ontario Council on Animal Care Committee.

RNA Isolation and RT-PCR

A nucleic acid isolation reagent (TRIzol; Life Technologies, Burlington, Ontario, Canada) was used to isolate total RNA as described previously.[22] First-strand cDNA synthesis was performed with reverse transcriptase (Superscript II system; Life Technologies). The resulting cDNA products were stored at −20°C. Real-time quantitative PCR was performed with fast cycling real time instrument (LightCycler; Roche Diagnostic Canada, Laval, Quebec, Canada) as described previously.[22] The data were analyzed by the standard curve method and normalized to 18S rRNA or β-actin mRNA. Primer sequences are given in the Table.

Immunofluorescence Staining and Western Blotting

We performed immunofluorescence staining for ED-B FN, CD31 (EC cell surface marker), and β1 integrin. Integrin antibody was obtained from Millipore, CD31 antibody from Santa Cruz Biotechnology (Santa Cruz, CA), and ED-B antibody was previously generated in our

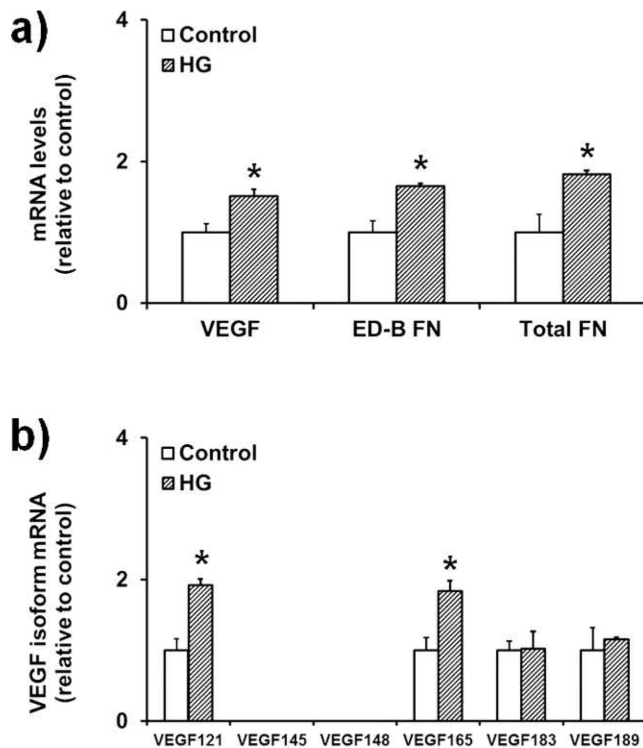

FIGURE 2. Expression of ED-B FN and VEGF in human retinal microvascular ECs (HRMECs). (**a**) Exposure of HRMECs to HG caused increased mRNA expression of VEGF, ED-B FN, and total FN compared to control glucose levels after 48 hours. *$P < 0.05$ compared to control. (**b**) RT-PCR analysis of VEGF isoform expression in HRMECs exposed to HG for 48 hours. *$P < 0.05$ compared to control.

laboratory.[22] After treating the cells with high glucose levels, we used methanol to fix the cells. Primary antibodies were used at 1:200 concentrations in PBS (containing 1% bovine serum albumin) and the cells were incubated for 1 hour at room temperature.

Alexa Fluor-488 and -594 conjugated secondary antibodies (Life Technologies) were used for detection (1:200 at room temperature) and DAPI (Vector Laboratories, Burlington, Ontario, Canada) for counterstaining. Images were taken using a compound fluorescent microscope (Olympus BX-51; Olympus Canada Inc., Richmond Hill, Ontario, Canada) equipped with a digital camera (Spot Pursuit; SPOT Imaging Solutions, Sterling Heights, MI).

For western blotting, we used 20 micrograms of total proteins per lane, resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and analyzed using VEGF and β-actin antibodies (Santa Cruz Biotechnology). The signals were detected with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and developed with the chemiluminescent substrate (Amersham Pharmacia Biotechnology, Amersham, UK).

Statistical Analysis

The data were expressed as means ± SEM. Differences were determined using ANOVA with post hoc Bonferroni's correction. *P* values < 0.05 were considered statistically significant.

RESULTS

High Glucose-Induced VEGF Expression Is Mediated by ED-B FN

We first investigated whether high levels of glucose alter the expression of VEGF in ECs and whether any change in VEGF was mediated by ED-B FN. A previous study has reported 40% induction in VEGF protein after 72 hours treatment with 30 mmol/L glucose.[23] We have shown that glucose increases FN mRNA and protein in as early as 24 hours in HUVECs.[22,27–29] Therefore, we exposed the cells to media containing 25 mmol/L glucose for 24 hours and assayed for VEGF transcript levels. Our results showed that HG increased VEGF mRNA levels as compared to cells exposed to 5 mmol/L glucose (Fig. 1a). An increase was also observed in ED-B containing FN mRNA levels. Twenty-five mmol/L L-glucose, used as osmotic control, did not produce any changes in VEGF mRNA levels (data not shown). These results showed that the newly expressed FN (induced by HG) predominantly contains ED-B. We hypothesized that the VEGF induction by high glucose levels is mediated by ED-B FN. To test this, we silenced ED-B FN in ECs and measured VEGF expression. If the hypothesis is true, we should see a reduction in glucose-induced VEGF expression upon ED-B FN knockdown. Our results showed that, indeed, HG-induced VEGF was completely normalized with ED-B siRNA transfection (Fig. 1a).

We next determined whether HG increases ED-B protein levels or localization by immunofluorescence staining. Our results showed no significant changes in ED-B FN fibril formation upon glucose challenge (Fig. 1b). However, the ED-B FN immunoreactivity was clearly higher in cells exposed to HG (images taken at the same exposure time).

Selective Upregulation of VEGF121 and VEGF165 by HG and ED-B Peptide

Alternative splicing of VEGF produces a number of isoforms that differ in their heparin– and heparin–sulfate-binding capacities.[30] We wanted to determine whether HG or ED-B peptide selectively upregulated certain VEGF isoforms. Therefore, we performed qPCR analysis (primer sequences presented in the Table; VEGF isoform sequences from Zygalaki et al.[31]) of major VEGF isoforms in ECs exposed to HG or ED-B peptide for 24 hours. Our results showed that both HG and ED-B peptide upregulated VEGF121 and VEGF165 isoforms (Fig. 1c). Other isoforms including VEGF145, 148, 183, and 189 showed no significant changes. It is possible that HG- or ED-B FN-induced VEGF may create a positive feedback loop to increase FN. In support of this notion are reports of VEGF increasing ECM proteins in ECs.[32] We treated ECs with recombinant VEGF165 and assayed for FN expression. Our results show a positive feedback loop where exogenous VEGF increased expression of total FN and ED-B containing FN (Fig. 1d).

Human Retinal Microvascular Endothelial Cells Show the Same Alterations as HUVECs

We have previously shown that ED-B FN is selectively increased in vitreous samples from patients with proliferative DR.[33] However, it remains to be determined whether HRMECs also increase ED-B FN expression upon exposure to high levels of glucose. We tested this possibility in cultured HRMECs and showed that with 48 hours of exposure to 25 mmol/L glucose, ED-B FN and VEGF expression was increased (Fig. 2a). Unlike HUVECs (Fig. 1), no changes were observed at 24 hours (data not shown). We also assayed for VEGF isoform expression in response to HG and found the same pattern as with HUVECs (Fig. 2b).

Inhibiting α2β1 and α5β1 Prevents Glucose- and ED-B FN Induced VEGF Expression

ECs express a number of integrin receptors.[34] These include 4x β1 (α1, α2, α3, and α5), αvβ3, and αvβ5 integrins. FN is

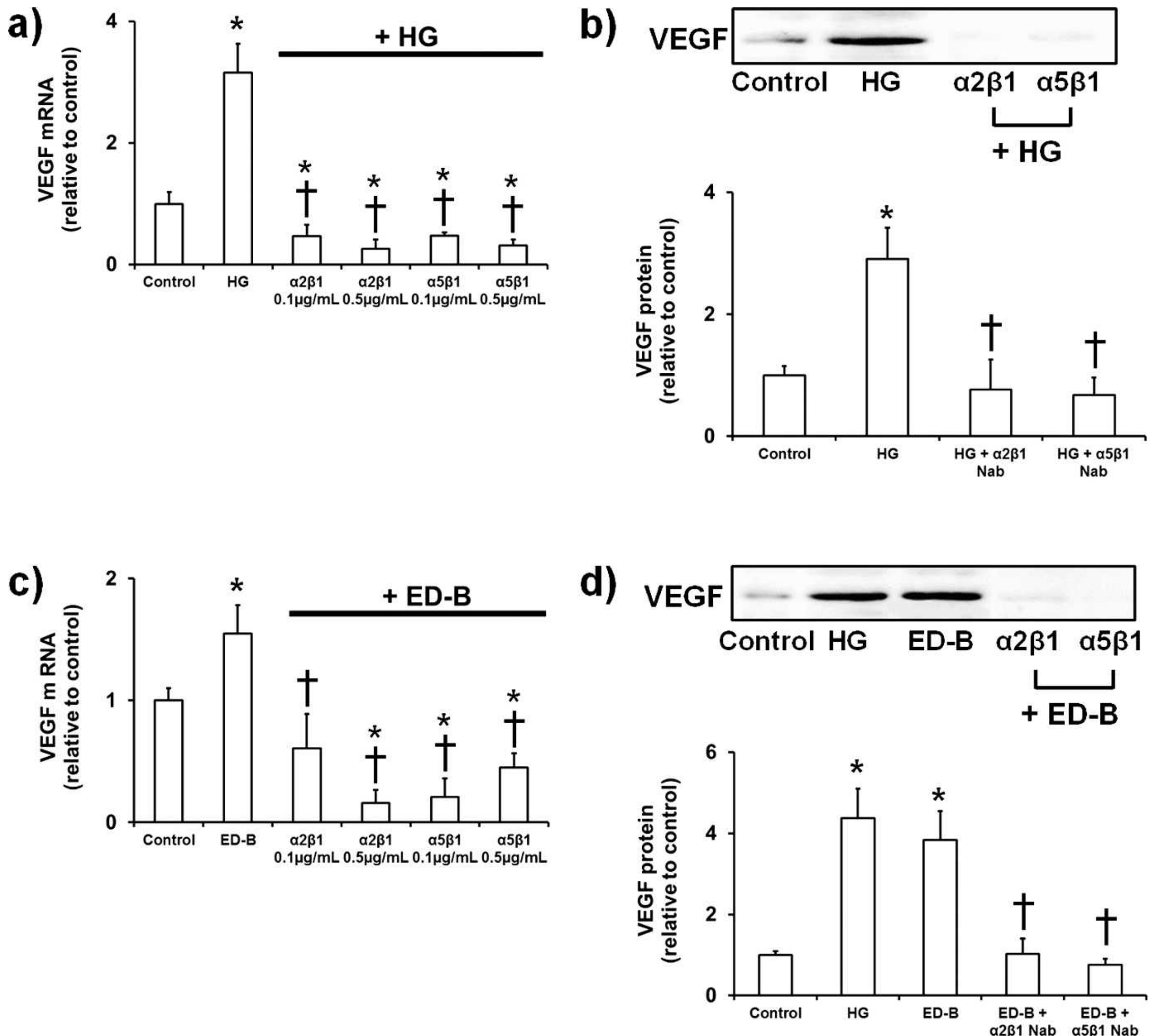

Figure 3. Effect of α2β1 and α5β1 integrin neutralizing antibodies on VEGF expression. HG caused increased mRNA expression (**a**) and protein production of VEGF (**b**) in HUVECs. Increased VEGF expression was also observed with ED-B peptide (**c**, **d**). Both glucose- and ED-B peptide-induced VEGF was inhibited by α2β1 and α5β1 antibodies. *$P < 0.05$ compared to control and †$P < 0.05$, compared to HG or ED-B peptide. For western blotting experiments, we used neutralizing antibodies at 0.5 μg/mL concentration.

recognized by α5β1 and $\alpha_V\beta3$. The remaining integrins expressed by ECs interact with other matrix proteins such as collagen (α1β1, α2β1), laminin (α1β1, α2β1, α3β1), and vitronectin (αvβ5). The sequence of FN recognized by α5β1 and $\alpha_V\beta3$ integrins is a tripeptide (Arg-Gly-Asp; RGD). In addition to the RGD site, α5β1 can also bind FN through a proximal site (32 Å; RGD).[35] We used a combination of antibodies and integrin-binding mimetics to determine whether ED-B FN increases VEGF expression through the engagement of integrins. Pretreatment of ECs with either α2β1 or α5β1 antibody prevented HG-induced and ED-B peptide-induced increase in VEGF mRNA and protein (Fig. 3). Pretreatment of HG- and ED-B peptide-exposed cells with IgG isotype control produced no changes at 0.5 μg/mL concentration (data not shown). Because of the limitation of reagents available for specific α-β integrin subunit combinations, we proceeded to test the involvement of integrins by using specific neutralizing antibodies against the β subunits (β1, β3, and β5) that inhibit the function of the integrin regardless of the α subunit partner. Our results showed that only β1 integrin neutralization prevents the increase in VEGF (Figs. 4a, 4b). Next, we determined whether β1 integrin inhibition would prevent VEGF expression by immobilized ED-B FN. To do this, we coated the culture plates with 1 μg/mL ED-B peptide and seeded the cells. Addition of β1 neutralizing antibody prevented VEGF expression (Fig. 4c). No changes were observed in cells treated with β3 or β5 neutralizing antibodies.

We wanted to know whether changes we saw in VEGF were due to altered subcellular distribution of β1 integrin. We then

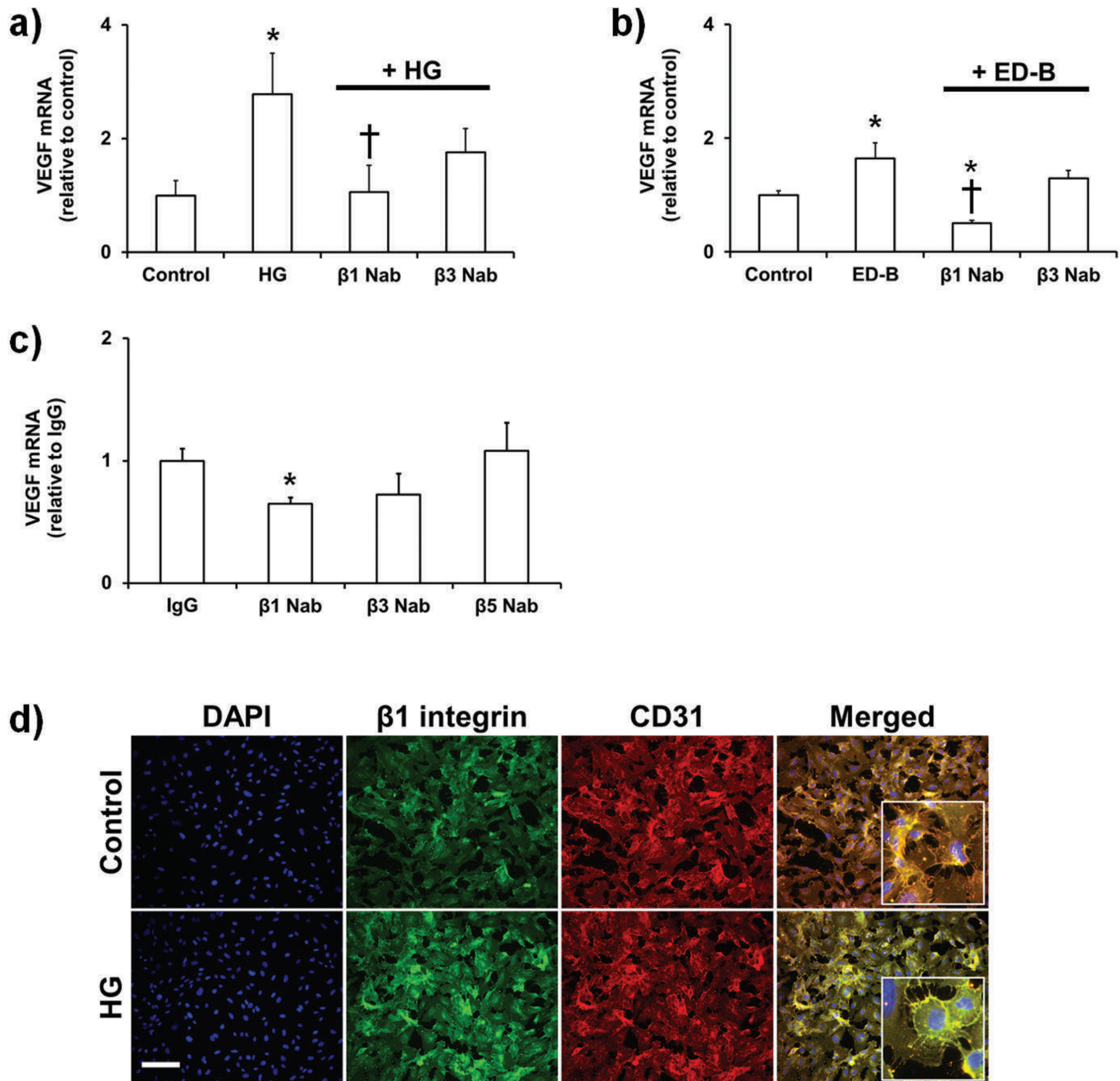

FIGURE 4. Involvement of β1 integrin in HG and ED-B FN mediated VEGF expression. VEGF expression by HG (**a**) or ED-B peptide (**b**) was inhibited by a specific neutralizing antibody against integrin β1. β3 neutralizing antibody had no significant effect. (**c**) Effect of the neutralizing antibodies on control (5 mmol/L glucose) cells plated on ED-B FN peptide coated plates (1 μg/cm^2). All neutralizing antibodies were applied at a concentration of 0.5 μg/mL. For (**a**, **b**), *$P < 0.05$ compared to control, †$P < 0.05$, compared to HG or ED-B peptide. For (**c**), *$P < 0.05$ compared to IgG control. (**d**) Immunofluorescence staining of β1 integrin in HUVECs exposed to HG (images taken at 20x; *scale bar* = 100 μm; inserts showing high power image; *green* = β1 integrin, *red* = CD31, and *blue* = DAPI).

performed immunofluorescence staining for β1 integrin in ECs exposed to HG. Our results showed no significant changes in the localization of β1 integrin upon HG exposure (Fig. 4d). Finally, we knocked down the expression of β1 integrin in ECs and observed similar results as with the neutralizing antibodies (Figs. 5a, 5b). Both HG- and ED-B peptide-mediated VEGF expression were completely normalized when β1 integrin was silenced.

So far, our results showed that HG increases ED-B FN, which then leads to VEGF expression through the β1 integrin. To show that these changes were mediated by direct binding of ED-B FN to integrin, we used integrin-binding peptides to competitively block the interaction between β1 integrin and ED-B FN. DGEA peptide is recognized by the α2β1 integrin, and the RGDS peptide is recognized by α5β1. Pretreatment with these peptides prevented HG-induced increase in VEGF mRNA and protein (Figs. 6a, 6b). In contrast, KDGE (scrambled peptide; negative control for DGEA) showed no effect on VEGF expression at the mRNA or protein level (Figs. 6a, 6b). As a functional readout of these effects, we assayed for tube

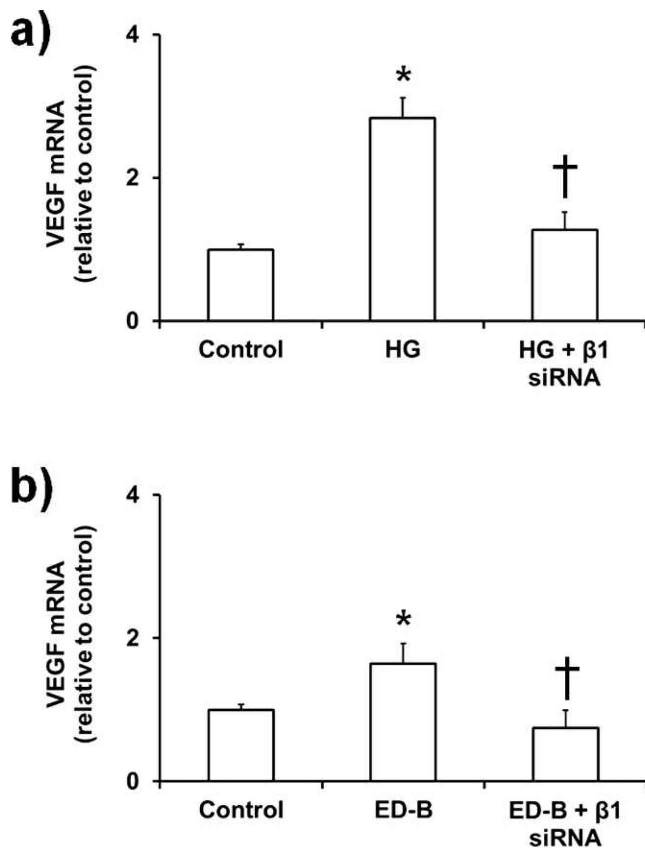

FIGURE 5. β1 silencing prevents VEGF expression. Silencing β1 integrin in HUVECs prevented both HG- (**a**) and ED-B peptide-induced (**b**) VEGF expression. $^{*}P < 0.05$ compared to control, and $^{\dagger}P < 0.05$, compared to HG or ED-B peptide.

formation by ECs. Exposure of ECs to HG increases tube morphogenesis (branching), which was prevented by DGEA and RGDS peptide, but not KDGE peptide (Fig. 6c). These results confirmed that the interaction between ED-B FN and α2β1/ α5β1 was necessary for the increased VEGF expression.

High Glucose Leads to Altered VEGFR2 Expression through ED-B Containing FN

We next determined whether HG altered the expression of VEGF receptors in ECs, and whether ED-B FN mediated these effects. Culture of ECs in HG significantly increased VEGFR2 expression (Fig. 7a), but not VEGFR1 (Fig. 7b). Since VEGFR1 has been shown to regulate the binding of VEGF to VEGFR2 (and thus the proliferative actions of VEGF),[36] these findings illustrate that HG increases VEGF-VEGFR2 signaling in ECs. Interestingly, we found that the HG-induced expression of VEGFR2 was mediated by ED-FN, as knockdown completely abolished VEGFR2 mRNA. VEGFR2 has been shown to cluster alongside integrins on the cell surface. It is possible that silencing ED-B FN modulates VEGFR2–integrin coupling and reduced VEGF expression.

Inhibiting ED-B FN in the Retina Prevents Diabetes-Induced β1 Integrin Expression and VEGF Production

In order to study whether the changes we observed in in vitro studies translate to the in vivo setting, we used an established animal model of chronic diabetes. We have previously shown that this STZ-diabetic model exhibits all molecular alterations reminiscent of human diabetes.[37–39] Although angiogenesis is not seen in animal models of diabetes, the molecular changes, including increased VEGF levels, have been reported by numerous laboratories. Here, we confirmed that 1 month of diabetes significantly increased VEGF expression in retinal tissues of diabetic mice (Fig. 8a). This increased VEGF was associated with augmented ED-B FN and β1 integrin. Because it is technically difficult to inhibit ED-B FN in the retina, we utilized systemic administration of ED-B siRNA. After administration of ED-B siRNA (every 7 days), we observed complete normalization of diabetes-induced ED-B expression in the retinal tissues. We then report that the augmented VEGF expression seen in the retinas of diabetic mice was normalized upon ED-B FN suppression. Treatment of nondiabetic animals with ED-B siRNA did not produce any significant effects (Fig. 8b). These findings indicated that diabetes led to VEGF expression in the retina through ED-B FN. Although we were not able to modulate β1 integrin levels directly, we showed that VEGF normalization, seen in the ED-B siRNA treated mice, was associated with reduced β1 integrin levels.

DISCUSSION

The salient findings of our study include (1) exposure of ECs to high levels of glucose increased VEGF through ED-B FN induction, (2) ED-B mediated the signals through β1 integrin, and (3) siRNA against ED-B FN decreased retinal VEGF and β1 integrin expression in diabetic mice. We also showed that ED-B peptide increased VEGF expression in ECs, which was inhibited by DGEA (containing the cell-binding domain of α2β1) and RGDS peptide (α5β1 binding peptide), but not KDGE peptide (without binding affinity for α2β1 integrin). The involvement of α2β1 and α5β1 was also confirmed by specific neutralizing antibodies and siRNA against β1 integrin.

The mechanisms of increased VEGF expression in DR may involve a direct role of glucose. Studies have shown that increased glucose levels upregulate VEGF expression in ECs,[23] smooth muscle cells,[40] and retinal pigment epithelial cells.[19,41,42] Interestingly, glucose deprivation may also increase VEGF expression and augment angiogenesis.[43] Whether this happens in ECs remains to be determined, but pericytes do show this response. It is possible that both mechanisms (elevated glucose as well as glucose deprivation/hypoxia) are at play in DR. Elevated glucose levels may cause EC damage and VEGF expression early on. This may lead to sustained expression levels of VEGF because of reduced perfusion and increased permeability. However, these further events need experimental support.

FN fragments have been shown to modulate EC adhesion and proliferation, and vasoregulation.[44–46] Studies have also shown that FN is upregulated in the active zones of retinal vascularization during development.[47] Since DR entails neovascularization in the late stages, it is expected that FN is also increased. In fact, the BM of diabetic animals do show elevated FN.[48] This happens as early as 1 month after diabetes onset in mice and rats.[33,49,50] In addition to increased production, the FN species are different in diabetes. We have recently demonstrated increased expression of ED-B FN in the vitreous samples of proliferative DR and retinal tissues of diabetic rats.[33] When we specifically inhibited ED-B FN in ECs, we observed decreased proliferation and tube morphogenesis.[22] This phenotypic change in the ECs was possibly due to changes in VEGF expression, as supported by the present study.

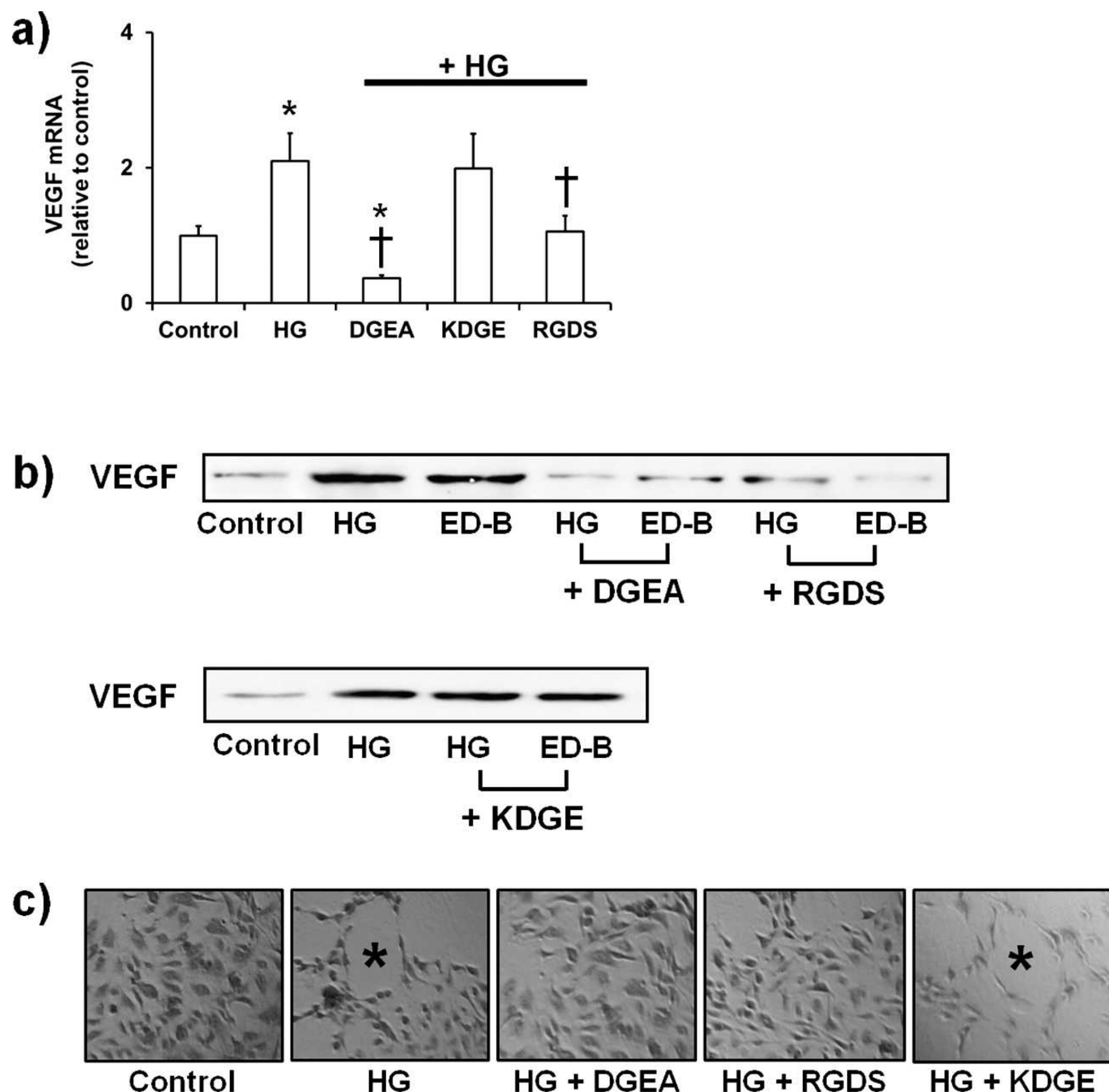

Figure 6. α2β1 and α5β1 integrin-binding peptides prevent VEGF expression and tube morphogenesis. DGEA and RGDS peptides, but not KDGE, prevented VEGF mRNA (**a**) and protein (**b**) expression in HUVECs. The cells were pretreated with the integrin-binding peptides (5 mmol/L) before exposure to HG or 2 μg/mL ED-B peptide. $^*P < 0.05$ compared to control, $^\dagger P < 0.05$, compared to HG. (**c**) Representative images of tube formation assay with or without pretreatment with the integrin-binding peptides (*asterisks* point to tube formation in HUVECs).

A novel finding of our study was the involvement of α2β1 integrin in ED-B mediated VEGF responses. This specific integrin (also known as VLA-2) has been shown to be a receptor for laminin and collagen.[51,52] Studies have also shown that antibodies directed against α2β1 inhibit adhesion of ECs to laminin- and collagen-coated surfaces, but have no inhibitory effects on FN-coated surfaces.[53] This raises a number of interesting questions regarding the mechanisms of ED-B mediated responses in ECs. These mechanisms may include modulation of collagen and/or laminin interaction with α2β1 by ED-B FN. ECs exposed to high levels of glucose increase collagen levels.[54] The presence of ED-B FN may modulate EC-collagen interaction leading to VEGF expression. Another possibility is that ED-B FN binds to α2β1 to cause VEGF expression. Interestingly, initial studies characterizing α2β1 have shown binding of this integrin to both plasma FN and a 110-Kd FN fragment (which contains the RGD site; close proximity to the ED-B segment).[55,56] The specificity of FN binding is similar to laminin and collagen IV.[55] Furthermore, this binding ability seems to be specific for ECs α2β1 integrin, as platelet-derived α2β1 did not bind FN.[56] As mentioned earlier, retinal pigment epithelial cells also induce VEGF expression in response to high levels of glucose. These cell types have been shown to express α2β1 integrin.[57] It would be interesting to determine whether retinal pigmental epithelial cell α2β1 integrin also binds to FN to mediate the high-glucose effect on VEGF expression.

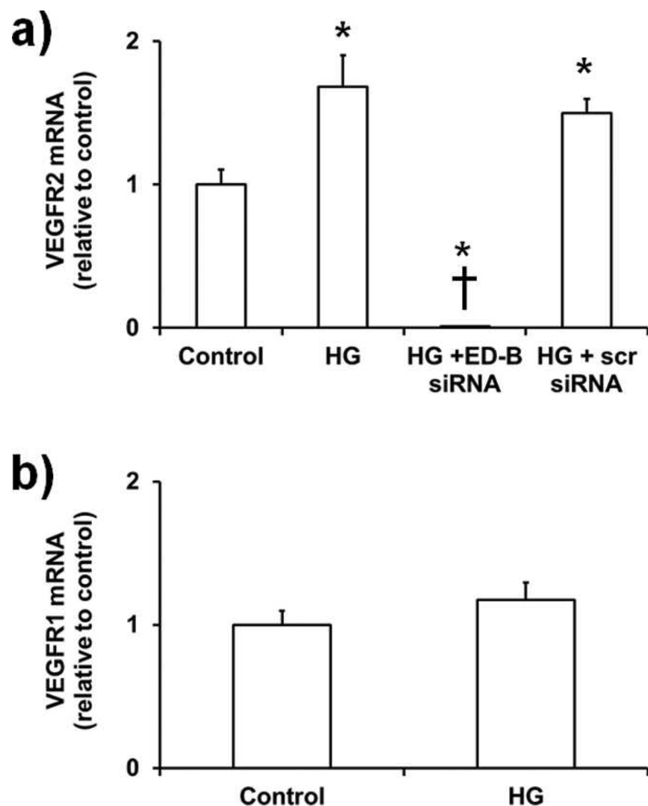

FIGURE 7. Alteration of VEGF receptor expression by HG in ECs. (**a**) Real-time PCR analysis of VEGFR2 expression in HUVECs exposed to HG. Silencing ED-B FN completely abolished the expression of VEGFR2. However, VEGFR1 (**b**) was not altered by HG. $^*P < 0.05$ compared to control, and $\dagger P < 0.05$, compared to HG.

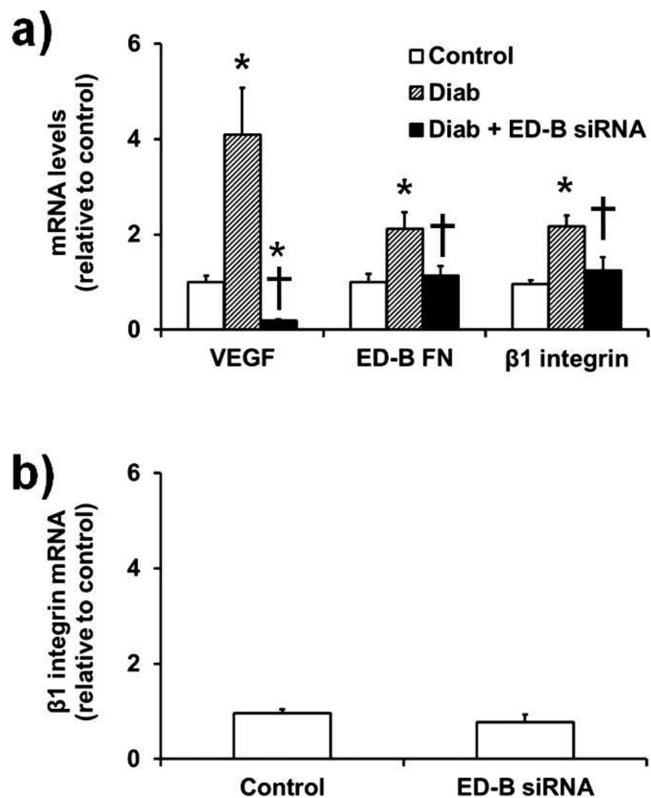

FIGURE 8. Effect of in vivo administration of ED-B siRNA on β1 integrin expression in the retina. (**a**) Real-time PCR analysis showing increased mRNA expression of integrin β1 in the diabetic mouse retina, which is prevented by systemic administration of ED-B FN siRNA. (**b**) No significant alteration in β1 integrin mRNA levels was observed when ED-B siRNA was administered to nondiabetic mice (control = nondiabetic; ED-B siRNA = nondiabetic treated with ED-B siRNA; Diab = diabetic treated with scrambled siRNA; Diab + ED-B siRNA = diabetic treated with ED-B siRNA. $^*P < 0.05$ compared to control and $\dagger P < 0.05$, compared to Diab.

An additional finding of our study was the regulation of VEGFR2 (but not VEGFR1) by ED-B FN. Knocking down ED-B in ECs completely abolished the expression of VEGFR2. This may be because of a positive feedback loop between VEGF and VEGFR2 expression.[58] Another possibility is that ED-B siRNA alters the cell membrane and causes VEGFR2 downregulation. VEGFR2 has been shown to interact with β1 integrin.[59] Neuropilin-1, a co-receptor of VEGFR1 and VEGFR2, also signals through α5β1 integrin in ECs.[60] Therefore, if ED-B siRNA caused changes in β1 integrin, VEGFR2 may be affected. Our results support this notion.

In summary, our results showed for the first time that high levels of glucose increased VEGF expression in ECs through the interaction of ED-B FN with β1 integrin. We have also shown that diabetes caused increased retinal VEGF and β1 integrin expression, which normalized upon ED-B FN inhibition. This represents a novel mechanism of autocrine VEGF expression and EC survival. EC-derived VEGF has previously been shown to be essential for cell survival through autocrine signals.[21] Mice with EC-specific VEGF knockout show systemic EC apoptosis. However, the same phenotype is not evident in the ED-B knockout animals. It is possible that in the ED-B knockout mice, a compensatory mechanism leads to VEGF expression from cell types adjacent to the ECs. Further studies are needed to determine whether ED-B FN induced VEGF expression is specific to ECs, or whether it represents a general biologic mechanism of VEGF expression. In addition, identifying the downstream signaling mechanisms may lead to new therapeutic targets for DR and other blood vessel–dependent diseases.

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