VEGF-initiated Blood–Retinal Barrier Breakdown in Early Diabetes

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PURPOSE. The objectives of this study were to (1) determine whether endogenous vascular endothelial growth factor (VEGF) triggers diabetic blood–retinal barrier breakdown, and (2) identify the site as well as phenotype of the hyperpermeable diabetic retinal vessels.

METHODS. Retinal VEGF mRNA levels were quantified in 1-week diabetic rats using the RNase protection assay. VEGF bioactivity was blocked via the systemic administration of a highly specific VEGF-neutralizing soluble Flt/Fc construct (VEGF TrapA40). An inactive IL6 receptor/Fc construct (IL6R Trap) was used as an isotype control. Blood–retinal barrier breakdown was quantified using the Evans blue technique and was spatially localized with fluorescent microspheres.

RESULTS. Retinal VEGF mRNA levels in 1-week diabetic animals were 3.2-fold higher than in nondiabetic controls (P < 0.0001). Similarly, retinal vascular permeability in 8-day diabetic animals was 1.8-fold higher than in normal nondiabetic controls (P < 0.05). Diabetes-induced blood–retinal barrier breakdown was dose-dependently inhibited with VEGF TrapA40, with 25 mg/kg producing complete inhibition of the diabetes-induced increases (P < 0.05). Blood–retinal barrier breakdown in diabetic animals treated with solvent alone or IL6R Trap did not differ significantly from untreated diabetic animals (P > 0.05). Spatially, early blood–retinal barrier breakdown was localized to the retinal venules and capillaries of the superficial retinal vasculature.


Diabetic retinopathy is a leading cause of blindness in middle-aged and older Americans.1 The single greatest source of vision loss in diabetes is macular edema,2 a pathology that is a direct consequence of diabetic blood–retinal barrier breakdown. An effective pharmacological treatment for this diabetic complication does not currently exist.

VEGF, a potent vasopermeability factor,3,4 may be operative in the pathogenesis of diabetic blood–retinal barrier breakdown. Retinal VEGF levels are upregulated in diabetes and coincide with blood–retinal barrier breakdown in rodents5,6 and humans.7 Flt-1 and Flk-1, the major high-affinity VEGF receptors, are similarly upregulated in the diabetic retina8 and localize to the retinal vasculature.9–10 Although VEGF is required for experimental retinal neovascularization,11 its definitive role in diabetic blood–retinal barrier breakdown is unknown.

The vessel phenotype(s) involved in blood–retinal barrier breakdown have not yet been determined. The blood–retinal barrier consists of two spatially distinct monolayers of cells: the retinal pigment epithelium (outer barrier) and the retinal vascular endothelium (inner barrier). Both monolayers possess tight junctions, which are presumably operative in the maintenance of the barrier. Although each monolayer exhibits increased permeability in diabetes, the retinal vasculature is the predominant site of leakage in early experimental diabetes12 and human diabetic retinopathy.13 Of note, VEGF receptors are present in the retinal vasculature but have not been detected in retinal pigment epithelium in vivo.14 At the cellular level, blood–retinal barrier breakdown is associated with endocytic vesicle formation and, to a lesser extent, degenerative endothelial changes.15

In the present study, the direct causal role of VEGF in diabetic blood–retinal barrier breakdown was analyzed. VEGF TrapA40, a high-affinity soluble VEGF receptor/Fc chimera, was administered systemically to diabetic animals to inhibit VEGF bioactivity and to assess its effect on blood–retinal barrier breakdown. In a separate set of experiments, the specific vasculature and vessel phenotype(s) responsible for diabetic blood–retinal barrier breakdown were identified using 0.1-μm red fluorescent microspheres. These microspheres label hyperpermeable blood vessels because they pass through the endothelial cell monolayer but are trapped in the vessel wall by the basal lamina (basement membrane). They do not, however, readily cross the endothelium of intact vessels.16
MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA), weighing approximately 200 g on arrival, were used in this study. The animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental procedures used aseptic sterile techniques and were approved by the Animal Care and Use Committee of the Children’s Hospital, Boston, MA.

Reagents

Evans blue dye (Sigma, St. Louis, MO) was dissolved in normal saline (30 mg/ml), sonicated for 5 minutes in an ultrasonic cleaner (G1125PIT; Laboratory Supplies, Hicksville, NY), filtered through a 5-μm filter (Millipore, Bedford, MA), and stored at 4°C. VEGF TrapA40 and IL6r Trap were synthesized by Regeneron Pharmaceuticals Inc. (Tarrytown, NY). VEGF TrapA40 was made from immunoglobulin repeats 1 to 3 of the extracellular domain of human Flt-1 fused to the Fc portion of human IgG. The protein was expressed in CHO cells and purified via protein A affinity chromatography, followed by size exclusion chromatography. The recombinant VEGF TrapA40 was then chemically modified to improve the pharmacokinetic profile of the parent molecule, without affecting its ability to bind VEGF with high affinity (Rudge J, Wiegand S, Yancopoulos GD, personal communication, 2000). The purity of the modified recombinant protein was determined to be >95% by Coomassie-stained SDS PAGE. The protein was filter sterilized and stored at 3.25 mg/ml in PBS, pH 7.2, containing 5% glycerol at -20°C.

IL6r Trap was similarly generated, using the extracellular domain of human IL6r alpha (the low-affinity IL6 receptor) fused to the Fc domain of human IgG. IL6r Trap only binds human IL6 with low affinity and does not bind to rat IL6. Like VEGF TrapA40, it was CHO cell derived, purified over protein A, and was >95% pure on Coomassie-stained gels. It was filter sterilized and stored at 3.25 mg/ml in PBS, pH 7.2, containing 5% glycerol at -20°C.

Experimental Diabetes

After a 24-hour fast, diabetes was induced with a single 60 mg/kg intraperitoneal injection of streptozotocin (Sigma) in 10 mM citrate buffer, pH 4.5. Animals that served as nondiabetic controls received an equivalent amount of citrate buffer alone. Twenty-four hours later, rats with blood glucose levels >250 mg/dl were deemed diabetic. Streptozotocin-injected rats with blood glucose levels <120 mg/dl were deemed “streptozotocin nondiabetic controls.” Just before experimentation, blood glucose levels were measured again to confirm diabetic status.

VEGF mRNA Protection Assay

Three normal nondiabetic and three 1-week diabetic rats were anesthetized with ketamine (80 mg/kg, Ketalar; Parke-Davis, Morris Plains, NJ) and xylazine (4 mg/kg, Rompun; Harver-Lockhart, Morris Plains, NJ). Both eyes were enucleated and bisected at the equator before they were killed. The retinas were then carefully dissected away and immediately frozen in liquid nitrogen until later use, at which time the tissue RNA was extracted.

Previously described templates for the transcription of rat VEGF and 18S riboprobes using RT-PCR were used. The RNase protection assay was performed as previously described. Briefly, a riboprobe for 18S rRNA (Ambion, Austin, TX) was included in each sample to control for variable loading and recovery of tissue RNA. Fifteen micrograms of total cellular RNA from both nondiabetic and 1-week diabetic rats was hybridized with 32P-labeled antisense VEGF and 18S rRNA riboprobes (200,000 cpm/probe) overnight at 42°C in 30 μl hybridization buffer. The riboprobe identified three major VEGF isoforms: VEGF120, VEGF164, and VEGF188. Hybridized RNA was digested with nuclease A (20 μg/ml; Ambion, Austin, TX) and RNase T1 (2 μg/ml; Ambion) for 1 hour at 25°C in 300 μl digestion buffer. The digestions were terminated via the addition of 20 μl of 10% SDS and 50 μg proteinase K (Ambion) for 15 minutes at 37°C. After acid guanidinium thiocyanate-phenol-chloroform extraction and ethanol precipitation, the protected fragments were resolved on 6% polyacrylamide, 7 M urea gels (Ambion) and visualized with autoradiography. Densitometry was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Administration of VEGF TrapA40

Ten 1-week diabetic rats received either 10 mg/kg (n = 5 animals) or 25 mg/kg (n = 5 animals) VEGF TrapA40 via tail vein injection. Ten 1-week diabetic controls received 25 mg/kg IL6r Trap (n = 5 animals) or an equivalent volume of solvent alone (PBS + 5% glycerol, n = 5 animals). Additional controls consisted of untreated diabetic (n = 3 animals), normal nondiabetic (n = 5 animals), and streptozotocin-treated nondiabetic animals (n = 5 animals).

Blood–Retinal Barrier Quantitation

Twenty-four hours after treatment, on day 8 of diabetes, the animals were anesthetized and the right jugular vein and right iliac artery were cannulated with 0.28- and 0.58-mm internal diameter polyethylene tubing (Becton Dickinson, Sparks, MD), respectively, and filled with heparinized saline (400 units heparin/ml saline). Evans blue was injected through the jugular vein over 10 seconds at a dosage of 45 mg/kg. Immediately after Evans blue infusion, the rats turned visibly blue, confirming their uptake and distribution of the dye. Subsequently, at 15-minute intervals, 0.1 ml blood was drawn from the iliac artery for 2 hours to obtain the time-averaged plasma Evans blue concentration, as described previously. After the dye had circulated for 120 minutes, the chest cavity was opened, and rats were perfused for 2 minutes via the left ventricle at 37°C with 0.05 M, pH 3.5, citrate-buffered paraformaldehyde (1% wt/vol; Sigma). The perfusion was at a physiological pressure of 120 mm Hg. A pH of 3.5 was used to optimize binding of Evans blue to albumin, and the perfusion solution was warmed to 37°C to prevent vasoconstriction. Immediately after perfusion, both eyes were enucleated and bisected at the equator. The retinas were carefully dissected away under an operating microscope and thoroughly dried in a Speed-Vac (Savant, St. Paul, MN) for 5 hours. The dry weight was used to normalize the quantitation of Evans blue leakage. Evans blue was extracted by incubating each retina in 120 μl formamide (Sigma) for 18 hours at 70°C. The supernatant was filtered through Ultrafree-MC tubes (30,000 NMWL, UFG3LTK00; Millipore, Bedford, MA) at 3000 rpm for 2 hours, and 60 μl of the filtrate was used for triplicate spectrophotometric measurements (Du640; Beckman, Fullerton, CA). Each measurement occurred over a 5-second interval, and all sets of measurements were preceded by known standards. The background-subtracted absorbance was determined by measuring each sample at both 620 nm, the absorbance maximum for Evans blue in formamide, and 740 nm, the absorbance minimum. The concentration of dye in the extracts was calculated from a standard curve of Evans blue in formamide. Blood–retinal barrier breakdown was calculated using the following equation, with results being expressed in μl plasmag retinal dry weight -1 h-1.

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\text{Evans blue (μg)/Retina dry weight (g)} = \frac{\text{Time-averaged Evans blue concentration (μg)/Plasma (μl)}}{\text{Circulation time (h)}}
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Spatial Localization of Diabetic Blood–Retinal Barrier Breakdown Using Fluorescent Microspheres

One-week diabetic rats and age-matched controls were anesthetized as described above and received a 0.2 ml tail vein injection of 0.1-μm red fluorescent microspheres (Duke Scientific, Palo Alto, CA), diluted fourfold in normal saline. Twenty minutes later, the chest cavity was
open under deep anesthesia with 50 mg/kg sodium pentobarbital. Lectin counterstaining of the vasculature was performed as described previously. Briefly, fixation was achieved via perfusion with 1% paraformaldehyde and 0.5% glutaraldehyde (Sigma) followed by perfusion with 5 mg/kg FITC-coupled Con A lectin (20 μg/ml in PBS, pH 7.4; Vector Laboratories, Irvine, CA). The lectin perfusion was followed by perfusion with PBS for 4 minutes. The retinas were then carefully dissected free and flat-mounted in a water-based fluorescence antifading medium (Southern Biotechnology, Birmingham, AL). The tissues were imaged with fluorescence microscopy (Zeiss Axiovert FITC filter; Zeiss, Wetzlar, Germany). Localization of leakage was performed by focusing up and down within the retinal flat mounts.

Statistics
Normally distributed data in two groups were analyzed with a Student’s t-test. For data that were not normally distributed, a Wilcoxon rank-sum test was used if there were two groups, and a Kruskal-Wallis test was used if there were three or more groups. All multiple pairwise comparisons used Duncan’s multiple range test. Differences were considered statistically significant if P < 0.05. All numerical results are expressed as means ± SE.

Results

Induction of Diabetes
The blood glucose levels (means ± SE; range) in nondiabetic animals and diabetic animals, just before experimentation, were 82 ± 3 mg/dl (range, 61–91 mg/dl) and 379 ± 9 mg/dl (range, 281–465 mg/dl), respectively (P < 0.0001, Student’s t-test). There were no changes in diabetic status compared with the first day postinduction of diabetes; that is, all diabetic animals remained diabetic.

Increased VEGF mRNA Levels in 1-Week Diabetic Retina

VEGF mRNA levels were 3.2-fold higher in 1-week diabetic rats than in normal nondiabetic rats (Fig. 1). The normalized VEGF mRNA levels (means ± SE) in nondiabetic animals and 1-week diabetic animals were 10.1 ± 0.33 optical density (OD) units (n = 5 retinas) and 32.2 ± 1.24 OD units (n = 6 retinas), respectively (P < 0.0001, Student’s t-test).

Suppression of 1-Week Diabetic Blood–Retinal Barrier Breakdown Using VEGF TrapA40

The retinal Evans blue leakage (median; range) in normal nondiabetic animals and STZ-treated nondiabetic animals was 5.31 (1.01–8.37; n = 10 retinas) and 4.02 (1.06–6.77; n = 10 retinas) µl plasmag retinal dry weight−1 h−1, respectively, demonstrating no significant difference between the medians of these two groups (Fig. 2A; P > 0.05, Wilcoxon rank-sum test). Because members of these two groups did not differ, they were combined into a single “nondiabetic” group (Fig. 2B; median, 4.86 µl plasmag retinal dry weight−1 h−1; range, 1.01–8.37; n = 20 retinas). The retinal Evans blue leakage (median; range) in untreated diabetic, PBS + glycercol-treated diabetic, and I6.8R Trap–treated diabetic rats was 8.55 (6.03–11.60; n = 6 retinas), 9.05 (6.56–13.00; n = 10 retinas), and 8.21 (5.67–12.69; n = 10 retinas) µl plasmag retinal dry weight−1 h−1, respectively, demonstrating no significant difference between the medians of these three control groups (Fig. 2A; P > 0.05, Kruskal–Wallis test). Because members of these three groups did not differ, they were combined into a single “diabetic” group (Fig. 2B; median, 8.69; range, 5.67 to 13.00; n = 26 retinas). The retinal Evans blue leakage in 1-week diabetic animals treated with 10 and 25 mg/kg VEGF TrapA40 was 6.38 (3.25–8.62; n = 9 retinas) and 4.03 (2.39–5.05; n = 9 retinas) µl plasmag retinal dry weight−1 h−1, respectively (Figs. 2A, 2B). A Kruskal–Wallis test confirmed that the medians of all four groups were significantly different from one another (P < 0.0001). Duncan’s multiple range test was then used to make multiple pair-wise comparisons. Retinal Evans blue leakage in diabetic rats was significantly higher than in nondiabetic animals (P < 0.05). The 10 and 25 mg/kg VEGF TrapA40–treated animals were significantly different from the diabetic controls (P < 0.05) as well as from each other (P < 0.05), supporting a dose-response effect. The 10 mg/kg, but not the 25 mg/kg, VEGF TrapA40 condition also differed significantly from the nondiabetic animals (P < 0.05).

Diabetic Blood–Retinal Barrier Breakdown in the Superficial Retinal Vasculature

The density and distribution of the red fluorescent microspheres in the vasculature of both normal nondiabetic and 1-week diabetic animals revealed marked differences. Nondia-
In the present study, retinal VEGF mRNA levels were measured in 1-week diabetic rats using an RNase protection assay and were found to be significantly higher than age-matched nondiabetic controls. Blood–retinal barrier breakdown temporally coincided with the increased retinal VEGF levels. VEGF TrapA40, a soluble Flt/Fc chimera, reversed early diabetic blood–retinal barrier breakdown in a dose-dependent manner. Moreover, VEGF TrapA40, at a dose of 25 mg/kg, restored diabetic blood–retinal barrier breakdown to nondiabetic levels. The effect was specific, as the administration of IL6R Trap, an inactive isotype control, had no effect on the same end point. The localization studies identified the retinal capillaries and venules of the superficial inner retinal vasculature as the primary sites of early blood–retinal barrier breakdown. The other half of the blood–retinal barrier, the RPE, was not analyzed in these studies.

The data indicate that VEGF is causal for early blood–retinal barrier breakdown in diabetes. These results must be reconciled with a recent report showing an 86% reduction in early diabetic blood–retinal barrier breakdown after ICAM-1 inhibition.22 The VEGF and ICAM-1 pathways are likely mechanistically linked. Exogenous VEGF stimulates ICAM-1 expression in the retinal vasculature,23,24 and VEGF recently was demonstrated to be an important endogenous inducer of ICAM-1 expression in the diabetic retina (Joussen AM, Poulaki V, Qin W, et al., personal communication, 2000). Moreover, ICAM-1 mediates leukocyte adhesion to the diabetic retinal vasculature,22 a process that initiates blood–retinal barrier breakdown. The mechanisms that underlie leukocyte-mediated vascular permeability are still not known. However, retinal endothelial cell injury and death may play a role, even after only 1 week of hyperglycemia.21 As noted above, diabetic blood–retinal barrier breakdown coincides with degenerative endothelial cell changes.13 If endothelial cell death is specifically prevented with an anti-FasL antibody, diabetic blood–retinal barrier dysfunction is also inhibited (Joussen AM, Poulaki V, Qin W, et al., personal communication, 2000). Leukocytes also have the ability to trigger the disorganization of endothelial cell-to-cell adherens25 and tight junctions26 in nonocular systems. Moreover, via their own VEGF, leukocytes may serve to amplify the direct permeability effects of VEGF. VEGF has been identified in diabetic animals showed only minimal entrapment of the red fluorescent microspheres in the walls of the major retinal venules (Figs. 3A, 3C). Diabetic animals, however, displayed significant trapping of the microspheres, predominantly in the venules and capillaries of the superficial inner retinal circulation (Figs. 3B, 3D). There was a distinct sparing of the major arteriolar circulation. The pattern of leakage in the major inner retinal venules was focal and homogeneous in nature. No evidence of microsphere trapping was seen in the deeper inner retinal vasculature of either the diabetic or nondiabetic animals (data not shown).

**DISCUSSION**

In the present study, retinal VEGF mRNA levels were measured in 1-week diabetic rats using an RNase protection assay and
neutrophils, 27 monocytes, 28 eosinophils, 29 lymphocytes 30 and platelets. 31 Fluorescent microspheres were used in these studies to localize leakage in the diabetic retina. These microspheres do not readily cross the endothelium of intact vessels but do label hyperpermeable blood vessels because they pass through the endothelial cell basal lamina but are trapped in the vessel wall by the basal lamina. In early diabetes, the venules and capillaries of the superficial inner retinal vasculature were demonstrated to be the principal sites of blood-retinal barrier breakdown. We speculate that the venular side of the vasculature is preferentially affected because the smooth muscle layer of the arteriolar circulation impedes the diffusion of VEGF made in the retinal parenchyma. Although McDonald and colleagues 16, 32 localized leakage in the tracheal mucosa after only 1 to 3 minutes of microsphere circulation, the present study required 20 minutes for optimal localization of retinal vascular leakage. The prolonged circulation time may be partially explained by the fact that unlike tracheal mucosa, the blood-retinal barrier has tight junctions, although they are breached in diabetes. 33 The breached diabetic blood-retinal barrier is still less permeable than leaky vessels elsewhere in the body. 18 Although fluorescent microspheres have no resemblance to any biological macromolecules, their usage was chosen over immunostaining for leakage of endogenous plasma proteins such as albumin, because unlike endogenous plasma proteins, particulate intravascular tracers, such as fluorescent microspheres, are trapped in the vessel wall and thus accumulate with the passage of time. This property has enabled fluorescent microspheres to be used successfully in the localization of sites of leakage in other systems. 16, 32 Admittedly, however, because the microspheres are larger than caveolae, they would be incapable of marking vascular leakage mediated by the caveolae membrane system.

Blood-retinal barrier breakdown in newly diabetic animals was also shown to be a consequence of diabetes itself and not an acute toxic effect of the diabetogenic drug streptozotocin. Retinal Evans blue content in normal non diabetic animals was compared with that of STZ-treated nondiabetic animals, that is, rats that had received STZ but remained nondiabetic, and the results were no different from normal nondiabetic animals. These data discount the notion of an acute toxic effect of STZ on retinal vascular permeability.

In conclusion, these experiments demonstrate that the inhibition of VEGF suppresses blood-retinal barrier breakdown in the superficial venules and capillaries of the inner retina, the principal site of vascular permeability in early diabetes. Taken together, the data identify a new molecular target for the treatment of early diabetic blood-retinal barrier breakdown.

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References


