

VEGF₁₆₄ Is Proinflammatory in the Diabetic Retina

Susumu Ishida,^{1,2,3} Tomobiko Usui,^{1,3,4} Kenji Yamashiro,^{1,5} Yuichi Kaji,^{1,4} Ednan Ahmed,¹ Karen G. Carrasquillo,^{1,6} Shiro Amano,⁴ Tetsuo Hida,⁷ Yoshihisa Oguchi,² and Anthony P. Adamis^{1,6}

PURPOSE. The objectives of this study were to characterize the differential potency of two major VEGF isoforms, VEGF₁₂₀ and VEGF₁₆₄, for inducing leukocyte stasis (leukostasis) within the retinal vasculature and blood-retinal barrier (BRB) breakdown and to determine whether endogenous VEGF₁₆₄ mediates retinal leukostasis and BRB breakdown in early and established diabetes.

METHODS. Retinal leukostasis and BRB breakdown were simultaneously quantified by combining concanavalin A lectin (ConA) perfusion labeling with a fluorophotometric dextran leakage assay. CD45 immunohistochemistry was performed to confirm that ConA-stained cells within the vasculature were leukocytes. Retinal leukostasis and BRB breakdown were compared in nondiabetic rats receiving intravitreal injections of VEGF₁₂₀ or VEGF₁₆₄. Retinal intercellular adhesion molecule (ICAM)-1 and VEGF protein levels were studied by Western blot and ELISA, respectively. An anti-VEGF₁₆₄₍₁₆₅₎ aptamer (EYE001) was administered by intravitreal injection to 2-week and 3-month diabetic rats, and the effect on retinal leukostasis and BRB breakdown was quantified.

RESULTS. Compared with VEGF₁₂₀, VEGF₁₆₄ more potently increased retinal ICAM-1 levels (2.2-fold), leukostasis (1.9-fold), and BRB breakdown (2.1-fold, $P < 0.01$ for all), despite negligible differences in vitreoretinal VEGF levels at the time of evaluation ($P > 0.05$). Retinal leukostasis and leakage increased with the duration of diabetes ($P < 0.01$) and correlated closely ($P < 0.01$, $r = 0.889$). The isoform-specific blockade of endogenous VEGF₁₆₄ with EYE001 resulted in a significant suppression of retinal leukostasis and BRB breakdown in both early (72.4% and 82.6%, respectively) and established (48.5% and 55.0%, respectively) diabetes ($P < 0.01$).

CONCLUSIONS. On an equimolar basis, VEGF₁₆₄ is at least twice as potent as VEGF₁₂₀ at inducing ICAM-1-mediated retinal leukostasis and BRB breakdown in vivo. The inhibition of

diabetic retinal leukostasis and BRB breakdown with EYE001 in early and established diabetes indicates that VEGF₁₆₄ is an important isoform in the pathogenesis of early diabetic retinopathy. (*Invest Ophthalmol Vis Sci.* 2003;44:2155-2162) DOI:10.1167/iov.02-0807

Diabetic retinopathy is frequently complicated by macular edema, a pathologic condition that is a direct consequence of blood-retinal barrier (BRB) breakdown. Macular edema can appear at any time during the course of diabetic retinopathy and is one of the greatest sources of vision loss in diabetes.¹ An effective pharmacological treatment for this complication of diabetes does not currently exist.

Vascular endothelial growth factor (VEGF) is a hypoxia-induced angiogenic factor^{2,3} and a vasopermeability factor.^{4,5} In patients with diabetic retinopathy, the VEGF levels in intraocular fluids increase not only during the proliferative stage,^{6,7} which is characterized by ischemia-initiated retinal angiogenesis, but also during the nonproliferative stage,^{8,9} during which hypoxia is less well documented. Furthermore, based on VEGF localization studies in surgically excised tissues from human diabetic eyes, VEGF has been suggested to play a crucial role both in fibrovascular proliferation^{10,11} and BRB breakdown.¹² The upstream stimuli for expression of VEGF in early retinopathy remain unknown.

Recently, it has been shown that leukocyte adhesion is operative in the pathogenesis of vascular leakage.¹³⁻¹⁵ VEGF increases the expression of intercellular adhesion molecule (ICAM)-1 on endothelial cells in vitro.^{16,17} In vivo, intravitreal injections of VEGF₁₆₄ induce ICAM-1 expression in the murine retinal vasculature.¹⁷ In a separate study, VEGF-induced BRB breakdown was shown to be leukocyte dependent in part, when the inhibition of ICAM-1 prevented BRB breakdown in VEGF₁₆₅-injected rat eyes.¹⁸

In the human retinal vasculature, leukocyte counts and ICAM-1 immunoreactivity are both increased in eyes with diabetic retinopathy.¹⁹ Similarly, experimental rat diabetes results in increased levels of retinal VEGF²⁰⁻²² and ICAM-1,²³ coincident with increased retinal leukocyte stasis (leukostasis),^{23,24} and BRB breakdown.^{20,23} When ICAM-1 bioactivity is inhibited with a neutralizing antibody, retinal leukostasis and BRB breakdown are both suppressed.²³ In experimental diabetes, the inhibition of VEGF suppresses retinal ICAM-1 expression,²⁵ leukostasis,²⁵ and BRB breakdown.²²

VEGF has at least five isoforms generated through the alternative splicing of mRNA arising from a single gene. The human proteins are one residue longer than the murine homologues. The two major prevalent isoforms in the retina are VEGF₁₂₁₍₁₂₀₎ and VEGF₁₆₅₍₁₆₄₎.²⁶ RNase protection assays have identified VEGF₁₆₄ as the predominant VEGF isoform expressed in the diabetic retina, accounting for at least 80% of total VEGF in experimental diabetes.²²

In the present study, the differential potency of VEGF₁₆₄ and VEGF₁₂₀ in inducing retinal leukostasis and BRB breakdown was compared. The current data demonstrate that on an equimolar basis, VEGF₁₆₄ was significantly more potent at inducing these pathologic responses when administered exogenously. Moreover, the inhibition of endogenous VEGF₁₆₄ po-

From the ¹Department of Ophthalmology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, Massachusetts; the ²Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan; the ³Department of Ophthalmology, Faculty of Medicine, University of Tokyo, Tokyo, Japan; the ⁴Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, Kyoto, Japan; ⁵Eyeteck Research Center, Woburn, Massachusetts; and the ⁶Kyorin Eye Center, Mitaka, Japan.

³Contributed equally to the work and therefore should be considered equivalent senior authors.

Submitted for publication August 8, 2002; revised November 8, 2002; accepted November 24, 2002.

Disclosure: **S. Ishida**, Eyeteck Pharmaceuticals (F); **T. Usui**, Eyeteck Pharmaceuticals (F); **K. Yamashiro**, Eyeteck Pharmaceuticals (F); **Y. Kaji**, Eyeteck Pharmaceuticals (F); **E. Ahmed**, Eyeteck Pharmaceuticals (F); **K.G. Carrasquillo**, Eyeteck Pharmaceuticals (E); **S. Amano**, None; **T. Hida**, None; **Y. Oguchi**, None; **A.P. Adamis**, Eyeteck Pharmaceuticals (E)

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Corresponding author: Anthony P. Adamis, Eyeteck Research Center, 42 Cummings Park, Woburn, MA 01801; tony.adamis@eyetec.com.

tently inhibited diabetic retinal leukostasis and BRB breakdown, both in early and established diabetes. Taken together, these data indicate that VEGF₁₆₄ is an appropriate target for the inhibition of several important diabetic retinal disorders.

METHODS

Induction of Experimental Diabetes

All animal experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care Committee of Massachusetts Eye and Ear Infirmary. After an overnight fast, Long-Evans rats (Charles River, Wilmington, MA), weighing 200 to 250 g, received single 60 mg/kg intraperitoneal injections of streptozotocin (Sigma, St. Louis, MO) in 10 mM citrate buffer (pH 4.5). Control nondiabetic animals received citrate buffer alone. Animals with blood glucose levels greater than 250 mg/dL 24 hours later were considered diabetic. The rats were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12-hour light-dark cycle until they were used for the experiments. Before each experiment, the diabetic state was reconfirmed. The animals selected for study had blood glucose levels greater than 250 mg/dL before death.

Intravitreal Administration of VEGF and the Anti-VEGF₁₆₅ Aptamer EYE001

Animals were anesthetized with intramuscular xylazine hydrochloride (6 mg/kg; Phoenix Pharmaceutical, St. Joseph, MO) and ketamine hydrochloride (40 mg/kg; Parke-Davis, Morris Plains, NJ). Intravitreal injections were performed by inserting a 33-gauge double-caliber needle (Ito Corp., Fuji, Japan) into the vitreous 1 mm posterior to the corneal limbus. Insertion and infusion were directly viewed through an operating microscope, taking care not to injure the lens or the retina. Any eyes that exhibited damage to the lens or retina were discarded and not used for the analyses.

Rats were randomized to receive intravitreal injections of 5 μ L of sterile phosphate-buffered saline (PBS) containing 1.7 pmol murine VEGF₁₂₀, VEGF₁₆₄ (R&D Systems, Minneapolis, MN), or vehicle alone. The dosage was determined from a previous report describing leukocyte adhesion to the rat retinal vasculature after intravitreal injections of VEGF₁₆₅.¹⁸ The retinas were analyzed 24, 48, and 72 hours after injection.

In separate experiments, diabetic animals received 5 μ L intravitreal injections of sterile PBS containing 2.5 nmol of the anti-VEGF₁₆₅ aptamer (EYE001; Eyetech Pharmaceuticals, New York, NY), 40-kDa polyethylene glycol (PEG; Eyetech), or vehicle alone. All reagents were injected 48 hours before evaluation. The anti-VEGF₁₆₅ aptamer is an oligonucleotide (28 ribonucleotide bases) that binds to the exon-7-encoded domain of human VEGF₁₆₅ with high specificity and affinity (200 pM).²⁷ It does not bind to VEGF₁₂₀₍₁₂₁₎. The oligonucleotide is conjugated to a 40-kDa PEG moiety to increase its half-life. The anti-VEGF₁₆₅ aptamer efficiently neutralizes VEGF₁₆₄ in rats,^{28,29} consistent with the fact that the exon-7-encoded VEGF domain is completely conserved between the rat and human. The dose and the evaluation time points were determined from a previous report.³⁰

Retinal Leukostasis Quantification

The retinal vasculature and adherent leukocytes were imaged with fluorescein-isothiocyanate (FITC)- or rhodamine-coupled concanavalin A lectin (ConA) (Vector Laboratories, Burlingame, CA). A perfusion labeling technique previously reported²⁴ was used, with slight modification. Animals were deeply anesthetized with intramuscular xylazine hydrochloride and ketamine hydrochloride. The chest cavity was carefully opened, and a 14-gauge perfusion cannula was introduced into the aorta. After drainage was achieved from the right atrium, the animals were perfused with 500 mL of PBS per kg body weight (BW) to remove erythrocytes and nonadherent leukocytes. Perfusion with

ConA (40 μ g/mL in PBS [pH 7.4], 5 mg/kg BW) was then performed to label adherent leukocytes and vascular endothelial cells, followed by removal of residual unbound lectin with PBS perfusion. The retinas were carefully removed, fixed with 1% paraformaldehyde, and flat-mounted in a mounting medium for fluorescence (Vector Laboratories). Each retina was imaged with an epifluorescence microscope (DM RXA; Leica, Deerfield, IL), and the total number of adherent leukocytes per retina was determined.

CD45 Immunofluorescence

Adherent leukocytes were labeled with FITC-coupled ConA, as just described. The flatmounted retinas were permeabilized with 0.5% Triton X (Sigma) in PBS for 24 hours, and nonspecific binding was blocked with 5% normal goat serum. The retinas were then incubated with a mouse anti-rat CD45 antibody (clone OX-1, 1:500; BD Pharmingen, San Diego, CA) overnight at 4°C, followed by incubation with a Texas red-conjugated goat antibody against mouse immunoglobulins (1:200; Jackson ImmunoResearch, West Grove, PA). The flatmounts were prepared with a mounting medium for fluorescence and then imaged with an epifluorescence microscope.

BRB Breakdown Quantification

After deep anesthesia with xylazine hydrochloride and ketamine hydrochloride, rats received intravenous injection of FITC-conjugated dextran (4.4 kDa, 50 mg/mL in PBS, 50 mg/kg BW; Sigma). After 10 minutes, the chest cavity was opened, and a 14-gauge perfusion cannula was introduced into the aorta. A blood sample was collected immediately before perfusion. After drainage was achieved from the right atrium, each rat was perfused with PBS (500 mL/kg BW) to clear the remaining intravascular dextran. The blood sample was centrifuged at 7000 rpm for 20 minutes at 4°C, and the supernatant was diluted at 1:1000. Immediately after perfusion, the retinas were carefully removed, weighed, and homogenized to extract the FITC-dextran in 0.4 mL of water. The extract was processed through a 30,000 molecular weight filter (Ultrafree-MC; Millipore, Bedford, MA) at 7000 rpm for 90 minutes at 4°C. The fluorescence in each 300- μ L sample was measured (excitation, 485 nm; emission, 538 nm), using a spectrofluorometer (SpectraMax Gemini XS; Molecular Devices, Sunnyvale, CA) with water as a blank. Corrections were made by subtracting the autofluorescence of retinal tissue from rats without FITC-dextran injection. The amount of FITC-dextran in each retina was calculated from a standard curve of FITC-dextran in water. For normalization, the retinal FITC-dextran amount was divided by the retinal weight and by the concentration of FITC-dextran in the plasma. BRB breakdown was calculated using the following equation, with the results being expressed in microliters per gram per hour

$$\frac{\text{Retinal FITC-dextran } (\mu\text{g})/\text{retinal weight (g)}}{\text{Plasma FITC-dextran concentration } (\mu\text{g}/\mu\text{L}) \cdot \text{circulation time (h)}}$$

The formula is identical with that used in the Evans blue dye leakage assay³¹ in recent reports.^{22,25,32,33}

The use of FITC-dextran in the quantification of BRB breakdown enabled the simultaneous assessment of retinal leukostasis. Perfusion labeling with rhodamine-ConA was used instead of PBS perfusion and was performed 10 minutes after the injection of FITC-dextran. Before homogenization, each retina was flatmounted, and the number of leukocytes counted under an epifluorescence microscope.

Western Blot Analysis for ICAM-1

Animals were killed with an overdose of anesthesia, and the retinas were immediately isolated. The retinas were subsequently homogenized in lysis buffer and centrifuged at 4°C for 10 minutes. The supernatants were collected and mixed with sample buffer. Each sample, containing 100 μ g of total protein, was then boiled for 3 minutes, separated by SDS-PAGE, and electroblotted to a polyvinyl-

dene difluoride (PVDF) membrane (BioRad, Hercules, CA). After non-specific binding was blocked with 5% normal goat serum, the membranes were incubated with a mouse anti-human ICAM-1 monoclonal antibody (1:200; Santa Cruz Biotechnologies, Santa Cruz, CA) at room temperature for 60 minutes, followed by incubation with a horseradish peroxidase-conjugated goat antibody directed against mouse immunoglobulin (1:20,000; Amersham Pharmacia, Piscataway, NJ). The signals were visualized with an enhanced chemiluminescence kit (ECL Plus; Amersham Pharmacia), according to the manufacturer's protocol.

Enzyme-Linked Immunosorbent Assay for VEGF

The animals were killed with an overdose of anesthesia, and the eyes were immediately enucleated. The retina-vitreous-lens capsule complex was carefully isolated and placed in 150 μ L of lysis buffer (20 mM imidazole HCl, 10 mM KCl, 1 mM MgCl₂, 10 mM EGTA, 1% Triton, 10 mM NaF, 1 mM sodium molybdate, 1 mM EDTA [pH 6.8]) supplemented with a protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) and sonicated. The lysate was centrifuged at 14,000 rpm for 15 minutes at 4°C, and the VEGF levels in the supernatant were determined with the mouse VEGF kit (Quantikine; R&D Systems), according to the manufacturer's protocol. The assay also recognizes rat VEGF. Total protein was determined using the bicinchoninic acid (BCA) kit (Bio Rad) and was used to normalize the VEGF protein levels.

Statistical Analyses

All results are expressed as the mean \pm SD. The data were processed for statistical analyses with the Mann-Whitney test, Spearman rank correlation, Kruskal-Wallis test, and Dunn procedure. Differences were considered statistically significant when $P < 0.05$. The percentage of inhibition was calculated, with the diabetes-induced increases representing 100%.

RESULTS

Correlation of Retinal Leukostasis and BRB Breakdown with the Duration of Diabetes

The retinal vasculature and adherent leukocytes were imaged with FITC-coupled ConA lectin in nondiabetic, 2-week diabetic, and 3-month diabetic rats. To confirm the identity of the ConA-labeled adherent cells, CD45 immunofluorescence detection was performed in retinal flatmounts. ConA/CD45 double labeling identified the adherent cells as CD45-positive leukocytes (Figs. 1A–C). The number of ConA-stained adherent leukocytes within the retinal vasculature increased with the duration of diabetes (Figs. 1D–F).

Rhodamine-coupled ConA perfusion labeling was combined with FITC-dextran spectrophotometry, allowing quantification of retinal leukostasis and BRB breakdown in the same retinas of nondiabetic, 2-week diabetic, and 3-month diabetic rats. The characteristics of the diabetic and control animals are summarized in Table 1. Age-matched control animals for early and established diabetes were used. Although the experiment was initiated with animals weighing 200 to 250 g, there were differences in final body weight between the age-matched control and the diabetic rats. As expected, the diabetic animals showed less weight gain. However, body weight did not appear to be a confounding variable, because retinal leukostasis and BRB breakdown did not differ between the control animals with early and established diabetes ($P > 0.05$) even though the mean body weight between the two groups was significantly different ($P < 0.01$).

Retinal leukostasis increased 2.1-fold in 2-week diabetic rats when compared with age-matched nondiabetic control animals (Fig. 1G, nondiabetes A; $P < 0.01$, Mann-Whitney test) and 3.3-fold in 3-month diabetic rats when compared with age-

matched nondiabetic control animals (Fig. 1G, nondiabetes B; $P < 0.01$). Similarly, BRB breakdown increased 2.2-fold in 2-week diabetic rats when compared with age-matched nondiabetic control animals (Fig. 1G, nondiabetes A; $P < 0.01$) and 6.4-fold in 3-month diabetic rats when compared with age-matched nondiabetic control animals (Fig. 1G, nondiabetes B; $P < 0.01$). The simultaneous quantification revealed a close correlation between retinal leukostasis and BRB breakdown (Fig. 1G; $P < 0.01$, $r = 0.889$, Spearman rank correlation) which increased significantly with the duration of diabetes.

Differential Potency of VEGF₁₂₀ and VEGF₁₆₄ in Inducing Retinal ICAM-1 Expression, Retinal Leukostasis, and BRB Breakdown

To examine the differential potency of the two major VEGF isoforms, exogenous murine VEGF₁₂₀ or VEGF₁₆₄ was injected into eyes of nondiabetic rats. VEGF ELISA measurements demonstrated that VEGF₁₂₀- and VEGF₁₆₄-injected eyes contained 99.4 ± 17.4 pg/mg versus 100.6 ± 15.4 pg/mg total VEGF at 24 hours after injection, 51.3 ± 4.2 pg/mg versus 51.6 ± 5.5 pg/mg at 48 hours, and 22.7 ± 5.4 pg/mg versus 32.3 ± 4.1 pg/mg at 72 hours, respectively ($n = 4$ each VEGF at each time point). The vitreoretinal VEGF protein levels measured 18.5 ± 1.3 pg/mg ($n = 4$) in the untreated eyes. The vitreoretinal VEGF protein levels did not differ at the 24- and 48-hour time points between the VEGF₁₂₀- and VEGF₁₆₄-injected eyes ($P > 0.05$, Mann-Whitney test).

As shown in Table 2, retinal leukostasis and BRB breakdown were quantified in normal rat eyes injected with vehicle, VEGF₁₂₀, or VEGF₁₆₄ at 24, 48, and 72 hours after injection, together with untreated eyes. Because both VEGF isoforms showed maximal effect on the retinal parameters at 48 hours, this was the time point chosen to compare the differential potencies of the two isoforms.

Western blot analysis for ICAM-1 (Fig. 2A) shows that retinal ICAM-1 protein levels 48 hours after intravitreal injection were elevated in the VEGF-injected eyes compared with the untreated and vehicle-injected eyes. Notably, retinal ICAM-1 levels were increased more potently by VEGF₁₆₄ than by VEGF₁₂₀ (2.2-fold difference, $P < 0.01$, Mann-Whitney test). Similar results were obtained in five separate experiments. Retinal leukostasis (Fig. 2B) was 1.9-fold higher at 48 hours in the VEGF₁₆₄- versus the VEGF₁₂₀-injected eyes ($P < 0.01$). BRB breakdown (Fig. 2C) was 2.1-fold higher at 48 hours in the VEGF₁₆₄- versus the VEGF₁₂₀-injected eyes ($P < 0.01$).

Suppression of Retinal Leukostasis and BRB Breakdown with the Anti-VEGF₁₆₄₍₁₆₅₎ Aptamer in Early and Established Diabetes

To investigate the effect of VEGF₁₆₄ isoform-specific antagonism, retinal leukostasis and BRB breakdown were assessed in eyes with early and established diabetes divided into four groups: untreated and vehicle-, PEG-, and anti-VEGF₁₆₅ aptamer-injected eyes. The characteristics of the nondiabetic control animals and early and established diabetic groups are summarized in Table 1. There was no difference in the plasma glucose levels among the four groups in early or established diabetes ($P > 0.05$, Kruskal-Wallis test). The untreated groups in Figures 3 and 4 are identical with the 2-week and 3-month diabetic eyes in Figure 1G.

The suppression of retinal leukostasis via VEGF₁₆₄-specific blockade is shown in Figure 3. Compared with PEG alone, treatment with the anti-VEGF₁₆₅ aptamer resulted in 72.4% inhibition of early diabetic retinal leukostasis (Fig. 3A, $P < 0.01$, Dunn procedure) and 48.5% inhibition of established diabetic retinal leukostasis (Fig. 3B, $P < 0.01$). The suppression of BRB breakdown after VEGF₁₆₄-specific blockade is shown in

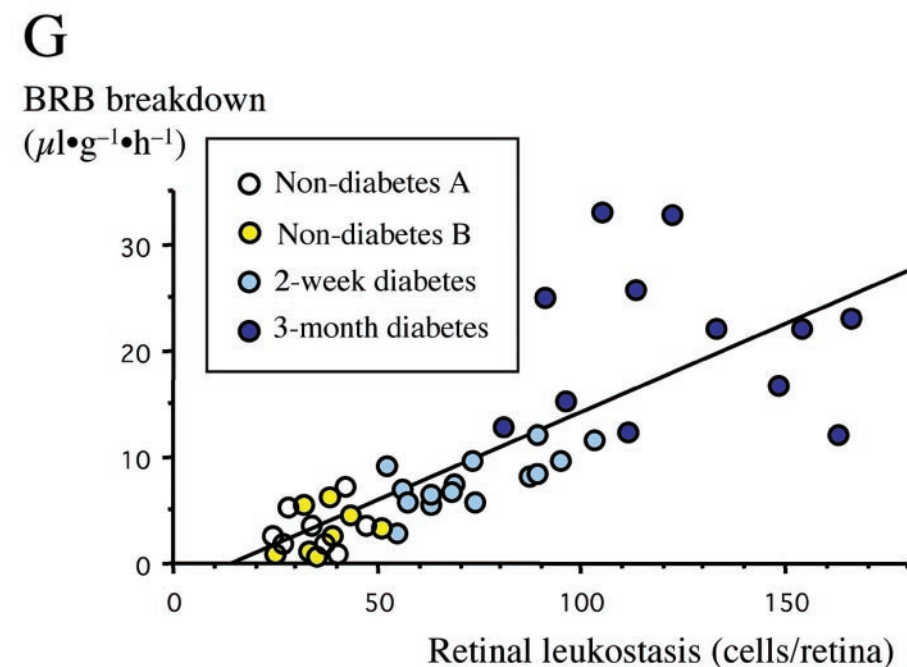
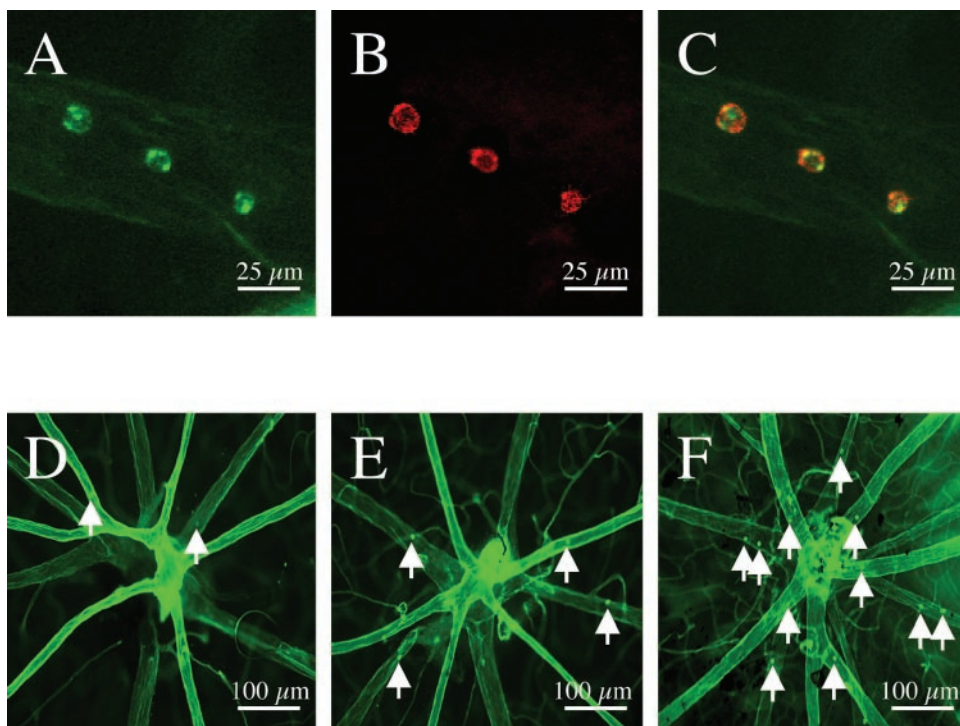


FIGURE 1. Correlation between diabetic retinal leukostasis and BRB breakdown. *Green* fluorescence from the FITC-coupled ConA (**A**) and *red* fluorescence from the anti-CD45 antibody (**B**) identified the ConA-stained cells as being CD45-positive leukocytes when the images were superimposed (**C**). The number of adherent leukocytes (*arrows*) within the retinal vasculature from nondiabetic (**D**), 2-week diabetic (**E**), and 3-month diabetic (**F**) animals increased with duration of diabetes. (**G**) There was a statistically significant correlation between retinal leukostasis and BRB breakdown ($P < 0.01$, Spearman's correlation coefficient = 0.889).

Figure 4. Compared with PEG alone, treatment with the anti-VEGF₁₆₅ aptamer resulted in 82.6% inhibition of early diabetic BRB breakdown (Fig. 4A, $P < 0.01$) and 55.0% inhibition of established diabetic BRB breakdown (Fig. 4B, $P < 0.01$).

DISCUSSION

The present study demonstrated the following new findings: (1) Diabetic retinal leukostasis and BRB breakdown, measured by a new method, increased with the duration of diabetes and correlated closely. (2) VEGF₁₆₄ was twice as potent as VEGF₁₂₀

at inducing retinal ICAM-1 expression, leukostasis, and BRB breakdown. (3) Endogenous VEGF₁₆₄ played a major role in the induction of diabetic retinal leukostasis and BRB breakdown.

The ability to measure both leukostasis and leakage in the same retina provided the opportunity to demonstrate that these two pathologic conditions correlated positively. Both also worsened as diabetes progressed. The data from the new combined procedure compare favorably and are in agreement with the data obtained using alternative methods, including scanning laser ophthalmoscopy,³⁴ FITC-ConA perfusion labeling,²⁴ the isotope dilution method,²³ and the Evans blue tech-

TABLE 1. Characteristics of Nondiabetic Control, Early, and Established Diabetic Rats

	Nondiabetes A (Age-Matched for 2-Week Diabetics)			2-Week Early Diabetes			Nondiabetes B (Age-Matched for 3-Month Diabetics)			3-Month Established Diabetes		
	Untreated	Vehicle	PEG	Untreated	Vehicle	PEG	Untreated	Vehicle	PEG	Untreated	Vehicle	PEG
Number	8	6	10	15	6	10	12	6	8	12	6	8
Body weight (g)	332 ± 35	241 ± 24	233 ± 18	239 ± 32	241 ± 24	233 ± 18	244 ± 19	360 ± 85	353 ± 72	373 ± 65	360 ± 85	353 ± 72
Blood glucose (mg/dL)	123 ± 18	348 ± 65	326 ± 55	359 ± 70	348 ± 65	326 ± 55	377 ± 82	420 ± 134	400 ± 121	445 ± 101	420 ± 134	400 ± 121
Retinal leukostasis (cells/retina)	34.9 ± 8.1	75.8 ± 13.7	79.9 ± 14.3	72.8 ± 16.1	75.8 ± 13.7	79.9 ± 14.3	47.3 ± 9.4*	126.8 ± 37.8	135.8 ± 31.8	123.6 ± 29.0	126.8 ± 37.8	135.8 ± 31.8
BRB breakdown μ L/g per h	3.43 ± 2.10	8.1 ± 2.62	7.91 ± 2.89	7.80 ± 2.46	8.1 ± 2.62	7.91 ± 2.89	4.21 ± 2.47*	18.90 ± 9.40	20.37 ± 7.50	21.10 ± 7.42	18.90 ± 9.40	20.37 ± 7.50

* Differences were examined among four groups in each diabetes by Kruskal-Wallis test and subsequent Dunn procedure as a post hoc test ($P < 0.01$).

TABLE 2. Differential Potency of Exogenous VEGF Isoforms for Inducing Retinal Pathologic Conditions

	24 Hours			48 Hours			72 Hours		
	Untreated	Vehicle	VEGF ₁₂₀	Untreated	Vehicle	VEGF ₁₂₄	Untreated	Vehicle	VEGF ₁₂₉
Number	7	4	7	8	6	8	8	4	6
Retinal leukostasis (cells/retina)	34.1 ± 9.4	38.0 ± 10.6	72.4 ± 23.9	113.4 ± 22.6	39.8 ± 13.5	81.7 ± 22.8	122.2 ± 35.3	32.6 ± 9.8	52.0 ± 9.0
BRB breakdown μ L/g per h	3.48 ± 2.18	3.34 ± 1.51	21.18 ± 7.60	49.69 ± 14.37	3.08 ± 1.34	30.03 ± 13.9	60.07 ± 20.91	3.50 ± 1.88	23.60 ± 10.04

At each time point, significant differences between VEGF₁₂₀ and VEGF₁₆₄ in both pathologic conditions were detected by Mann-Whitney test ($P < 0.01$).

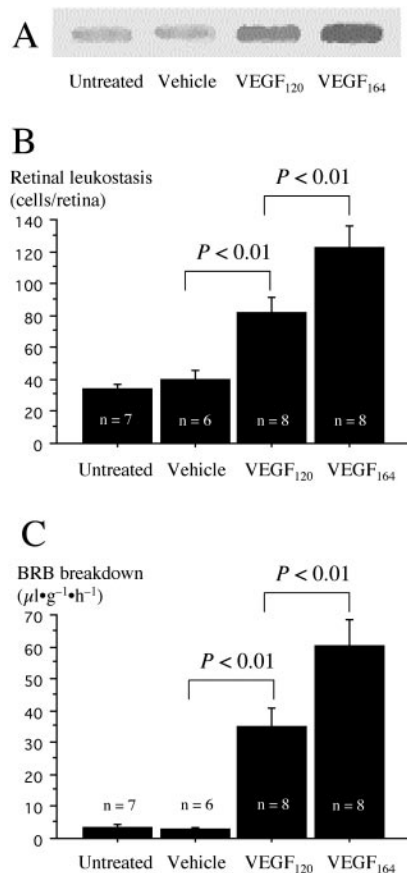


FIGURE 2. Differential induction of retinal leukostasis and BRB breakdown at 48 hours with VEGF₁₂₀ and VEGF₁₆₄. (A) Western blot analysis for ICAM-1 showed that retinal ICAM-1 was upregulated by the VEGF isoforms, more potently by VEGF₁₆₄ than VEGF₁₂₀. (B) VEGF₁₆₄ induced a 1.9-fold greater increase in retinal leukostasis than did VEGF₁₂₀ ($P < 0.01$). (C) VEGF₁₆₄ induced a 2.1-fold greater increase in BRB breakdown than did VEGF₁₂₀ ($P < 0.01$).

nique.³¹ Previous studies have used morphometry to assess dextran leakage and BRB breakdown in tissue sections.^{35,36} The current method extends those capabilities by providing a level of overall sensitivity and quantitation comparable to that of the Evans blue dye leakage assay.^{22,25,31-33}

Previous work has shown that VEGF₁₆₅ is a more potent endothelial cell mitogen than VEGF₁₂₁.^{37,38} This is explained in part by the recent evidence that neuropilin-1, a VEGF₁₆₅ receptor that binds to the exon-7-encoded domain lacking VEGF₁₂₁, enhances VEGF receptor (R)-2 signal transduction,^{39,40} which is responsible for VEGF-induced mitogenesis.^{41,42} VEGF₁₆₅ is also thought to facilitate angiogenesis in human diseases when it is coexpressed with VEGFR-2 and neuropilin-1.^{11,43} The present study demonstrates for the first time that VEGF₁₆₄ is more potent at inducing BRB breakdown than VEGF₁₂₀. This result may be explained by the recent finding that VEGFR-2, but not VEGFR-1, stimulation is responsible for vascular permeability⁴⁴ and endothelial cell mitogenicity.^{41,42}

The present study documents that VEGF₁₆₄ induces retinal leukostasis and BRB breakdown more potently than VEGF₁₂₀. A previous report showed that VEGF-induced BRB breakdown is mediated, in part, through ICAM-1-dependent retinal leukostasis.¹⁸ New in vitro and in vivo data also show that VEGF₁₆₅ more potently induces endothelial ICAM-1 expression, as well as leukocyte adhesion and migration (Usui T, Ishida S, Yamashiro K, et al., manuscript submitted, 2002). In eyes with

early diabetes, the expression of retinal VEGF₁₆₄ is 11 times higher than VEGF₁₂₀.²² The current data show that in addition to this differential expression, VEGF₁₆₄, on an equimolar basis, was approximately two times more potent in inducing leukostasis and BRB breakdown than VEGF₁₂₀. Thus, VEGF₁₆₄ is more proinflammatory in the retina than is VEGF₁₂₀.

The current data also show that an anti-VEGF₁₆₅ aptamer can suppress retinal leukostasis and BRB breakdown in both early and established diabetes. The efficacy in established diabetes was diminished but still notable. We speculate that this is attributable, in part, to the accumulation of advanced glycation end products (AGEs). A significant increase in retinal AGE levels is seen at 3 months of diabetes and is not present at 2 weeks (Kaji Y, Ishida S, Yamashiro K, et al., unpublished data, 2002). AGEs can directly induce vascular endothelial ICAM-1, both in vivo⁴⁵ and in vitro.⁴⁶ This direct stimulation of ICAM-1 gene expression by AGEs probably dilutes the stimulatory effect of VEGF over time.

A soluble VEGFR-1/Fc fusion protein that inhibits all VEGF isoforms has been shown to suppress retinal leukostasis²⁵ and leakage efficiently²² in early diabetes. The degree of inhibition observed with the anti-VEGF₁₆₅ aptamer in the present study was comparable to the inhibition achieved with the VEGFR-1/Fc fusion protein. The comparability of the results with the two VEGF inhibitors is consistent with our hypothesis that VEGF₁₆₅, but not VEGF₁₂₁, plays a major role in diabetic retinal leukostasis and BRB breakdown. As the disease progresses to proliferative diabetic retinopathy, VEGF₁₆₅-specific signaling is thought to accelerate fibrovascular proliferation.¹¹ Considering that VEGF and its receptors are constitutively expressed in the

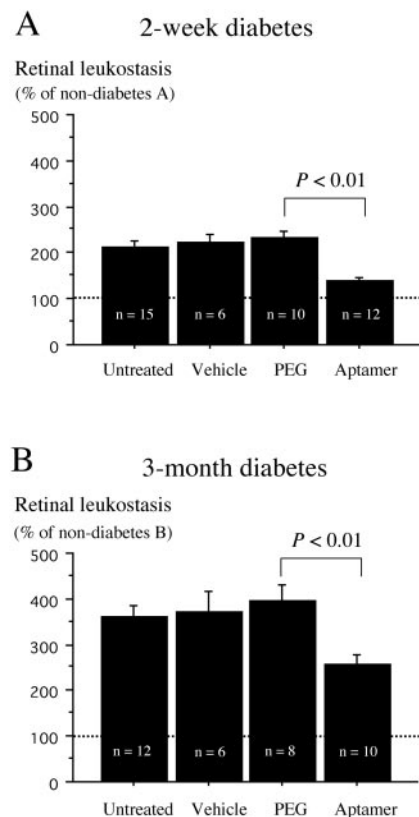


FIGURE 3. Suppression of diabetic retinal leukostasis by the anti-VEGF₁₆₅ aptamer. Compared with PEG alone, treatment with the anti-VEGF₁₆₅ aptamer EYE001 resulted in (A) 72.4% blockade of early diabetic retinal leukostasis ($P < 0.01$) and (B) 48.5% blockade of established diabetic retinal leukostasis ($P < 0.01$).

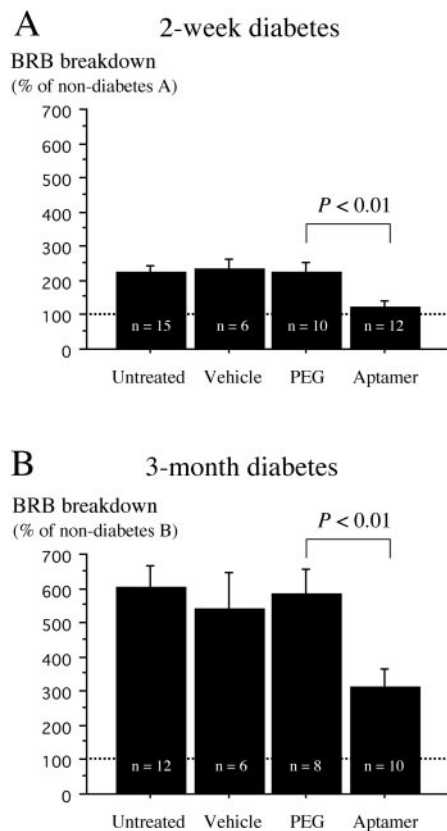


FIGURE 4. Suppression of diabetic BRB breakdown by anti-VEGF₁₆₅ aptamer. Compared with PEG alone, treatment with the anti-VEGF₁₆₅ aptamer EYE001 resulted in (A) 82.6% blockade of early diabetic BRB breakdown ($P < 0.01$) and (B) 55.0% blockade of established diabetic BRB breakdown ($P < 0.01$).

normal retina,²⁶ the complete blockade of VEGF bioactivity may therefore not be desirable.⁴⁷ Several reports have recently demonstrated the neuroprotective effects of VEGF both in vivo⁴⁷ and in vitro.^{48–50} Thus, blocking VEGF₁₆₅ alone, a more pathogenic isoform in the retina, may be more desirable.

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