Altered Expression Patterns of VEGF Receptors in Human Diabetic Retina and in Experimental VEGF-Induced Retinopathy in Monkey

Antonella N. Witmer,1,2 Harriet G. Blaauwgeers,1 Herbert A. Weich,3 Kari Alitalo,4 Gijs F. J. M. Vrensen,2 and Reinier O. Schlingemann1

PURPOSE. The vascular endothelial growth factor (VEGF) family is involved in vascular leakage and angiogenesis in diabetic retinopathy (DR) in the eye, but may also have physiological functions. Based on the hypothesis that differential VEGF receptor (VEGFR) expression in the retina is an important determinant of effects of VEGF, this study was conducted to investigate VEGFR expression in the diabetic retina and in an experimental monkey model of VEGF-induced retinopathy.

METHODS. In retinas of 27 eyes of diabetic donors, 18 eyes of nondiabetic control donors, and 4 monkey eyes injected with PBS or VEGF-A, expression patterns of VEGFR-1, -2, and -3 in relation to leaky microvessels, as identified by the marker pathologische anatomie Leiden-endothelium (PAL-E) were studied by immunohistochemistry.

RESULTS. In control human retinas and retinas of PBS-injected monkey eyes, all three VEGFRs were expressed in nonvascular areas, but only VEGFR-1 was constitutively expressed in retinal microvessels. In diabetic eyes, increased microvascular VEGFR-2 expression was found in association with PAL-E expression, whereas microvascular VEGFR-3 was present in a subset of PAL-E-positive cases. In VEGF-A-injected monkey eyes, VEGFR-1, -2, and -3 and PAL-E were expressed in retinal microvessels.

CONCLUSIONS. The VEGFR-1, -2, and -3 expression patterns in control retinas suggest physiological functions of VEGFs that do not involve the vasculature. Initial vascular VEGF signaling may act primarily through VEGFR-1. In diabetic eyes, expression of retinal VEGFR-2 and -3 is increased, mainly in leaky microvessels, and VEGF-A induces vascular expression of the VEGF-A receptor VEGFR-2 and the VEGF-C/D receptor VEGFR-3. These findings indicate a dual role of VEGFs in the physiology and pathophysiology of the retina and suggest that microvascular VEGFR-2 and -3 signaling by VEGFs occurs late in the pathogenesis of DR, possibly initiated by high levels of VEGF-A in established nonproliferative DR. (Invest Ophthalmol Vis Sci. 2002;43:849–857)

Vascular endothelial growth factor (VEGF)-A is an important cytokine involved in the pathogenesis of diabetic retinopathy (DR). It is a member of the VEGF family, together with VEGF-B, -C, and -D and placenta growth factor (PlGF). VEGFs can bind to three distinct tyrosine kinase receptors: VEGFR-1 (Flt-1), -2 (KDR), and -3 (Flt-4). Ligands of VEGFR-1 include VEGF-A and -B and PlGF; ligands for VEGFR-2 are VEGF-A, -C, and -D; and ligands for VEGFR-3 are VEGF-C and -D. VEGFRs mediate various cellular functions. After binding of a ligand, VEGFR-1 signaling leads to migration of endothelial cells (and of monocytes-macrophages), production of proteases, and expression of tissue factor, whereas ligand binding of VEGFR-2 on endothelial cells causes nitric oxide production, increased permeability of blood vessels, and proliferation of endothelial cells. VEGF-2 signaling is crucial in angiogenesis, and its expression in activated endothelium correlates strongly with expression of one of its ligands, VEGF-A, whereas it is low or absent in quiescent endothelium. VEGFR-2 expression is up-regulated by hypoxia and possibly also by VEGF-A itself. In addition, it has been suggested that production of a soluble form of VEGFR-1 (sVEGFR-1) is also regulated by hypoxia. After binding of VEGF-A, sVEGFR-1 counteracts VEGF-A activity and thus participates in regulation of angiogenesis.

Recent literature on the possible role of VEGF in ocular angiogenesis and DR is complex. On the one hand, extensive evidence is available suggesting that VEGF is the main cytokine causing vascular leakage and angiogenesis in various conditions involving retinal ischemia. In nonproliferative DR, leakage of retinal microvessels is probably caused by local VEGF production in areas of capillary nonperfusion. In addition, VEGF mRNA levels are increased in the ischemic retina of patients with advanced DR, and proliferative DR is associated with high levels of VEGF in the vitreous. On the other hand, various reports show mRNA and protein expression of VEGF-A and VEGFR-1 and -2 in the normal retina, suggesting physiological functions of VEGF.

Because differential expression of its receptors on vascular cells may be an important mechanism of regulating the activity of VEGF, it has been hypothesized that distribution patterns and/or levels of VEGFR are major determinants of VEGF activity in the normal retina or in pathologic conditions such as DR. However, few data are available on expression patterns of VEGFRs in the retinal vasculature of control or DR eyes. It is also unknown how VEGF expression in the retina is regulated. Increased VEGFR-2 expression has been described in relation to retinal ischemia in mice, and in background DR in rats, both inside and outside the vasculature. VEGFR-2 and -3 expression in human retinal microvessels is associated with proliferative DR. However, these studies did not include histologic markers of vascular changes related to DR. We have recently shown that local loss of the blood-retinal barrier and specific morphologic changes in capillaries...
in DR are highly correlated with the expression of the antigen *pathologische anatomie Leiden-endothelium* (PAL-E), a marker for nonbarrier endothelium. Expression of PAL-E and staining of permeability markers were found in specific areas in a pattern similar to that of DR lesions in clinical angiography. Proliferative DR caused widespread staining of PAL-E in retinas. Therefore, the PAL-E antigen can be considered an immunohistochemical marker of areas with vascular leakage related to DR.

In the present study, we investigated (1) the expression patterns of VEGFRs in retinas of control and diabetic eyes, in relation to staining of both the entire vasculature using CD31 antibodies and leaky microvessels using the antibody PAL-E, and (2) the expression patterns of VEGFRs in retinal microvessels in a model of VEGF-induced retinopathy in monkeys.

**MATERIALS AND METHODS**

**Human Eyes**

Eyes of 18 persons without and 27 persons with diabetes mellitus (for reasons of privacy, information on the type of diabetes mellitus was not available).
available) were obtained from the Corneabank Amsterdam (The Netherlands), after removal of corneal buttons for transplantation. The age-range of control donors was between 51 and 76 years (mean, 69 years) and the time since death ranged between 8 and 33 hours (mean, 23 hours). The age-range of diabetic donors was between 54 and 84 years (mean, 73 years) and time since death ranged between 6 and 32 hours (mean, 18 hours). Intact eyes were snap frozen in isopentane and stored at $-70^\circ$C until used. The use of human tissue was in accordance with the tenets of the Declaration of Helsinki regarding the use of human tissue for research.

**Monkey Eyes**

Two cynomolgus monkeys (*Macaca fascicularis*), a 15-year-old male and a 5-year-old female, were used for the experiments. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in accordance with the guidelines for animal care at the University of Nijmegen, The Netherlands. Both animals had been used for behavioral studies unrelated to visual function and without alteration of the eyes. The animals received four injections of phosphate-buffered saline (50 $\mu$L PBS, pH 7.4) in the left eye through the pars plana into the center of the vitreous, with a 30-gauge needle (at days 0, 2, 4, and 7), and four injections with bioactive human recombinant VEGF-A (0.5 $\mu$L PBS; Harbor Bio-Products, Norwood, MA) in the right eye on the same days. Before the intravitreal injections, 20 mg/kg ketamine hydrochloride, 0.005 mg/kg acepromazine, and 0.03 mg/kg atropine sulfate were administered intramuscularly for general anesthesia. Ten minutes after injection,
intraocular pressure was measured using a Schiotz tonometer (Sklar Manufacturing, New York, NY). The animals were killed at day 9 with an overdose of intravenous pentobarbital and subsequently perfused through the abdominal aorta with PBS (37°C, pH 7.4) for 10 minutes at a perfusion pressure of 70 to 80 mm Hg before the eyes were enucleated. Eyes were dissected, and the posterior segment was snap frozen in liquid nitrogen and stored at −70°C until used.

Immunohistochemistry

Tissue blocks of the posterior half of the frozen human and monkey globes were cut according to a standard protocol. Air-dried serial cryostat sections (10 μm thick) of one tissue block containing the midperipheral retina and posterior pole of one eye of each patient and of both eyes of both monkeys were fixed in cold acetone for 10 minutes, postfixed for 2 minutes in Zamboni fixative (2% paraformaldehyde in a saturated picric acid solution), and stained by an indirect immunoperoxidase procedure. For this purpose, sections were incubated for 20 minutes in PBS containing 0.1% sodium azide and 0.03% H2O2 to quench endogenous peroxidase activity. To reduce nonspecific staining, sections were incubated with a broad-spectrum serum blocking solution (Histostain Plus kit; Zymed, San Francisco, CA) in PBS containing 0.05% saponin (Sigma, St. Louis, MO) for 15 minutes. Subsequently, sections were incubated overnight at 4°C with a solution of the after antibodies: monoclonal antibodies Flt-19 (against VEGFR-1, 1:400) and KDR-1 (against VEGFR-2, 1:400),34 monoclonal antibody 9D9F9 (against VEGFR-3, 1:1500),35 and the anti-endothelial monoclonal antibodies PAL-E (1:1000)36 and EN-4 (against CD31, 1:500).37 Flt-19 and KDR-1 were kindly provided by Herbert A. Weich, National Research Center for Biotechnology, Braunschweig, Germany; 9D9F9 was provided by Kari Alitalo, Haartman Institute, Helsinki, Finland; and EN-4 by Sanbio, Uden, The Netherlands. For negative control incubations, primary antibody was omitted, or sections were stained with an antibody against a nonhuman bacterial protein (mouse negative control immunoglobulins; Dako, Glostrup, Denmark). Sections were subsequently incubated with biotinylated goat anti-mouse immunoglobulins for 15 minutes, followed by streptavidin-horseradish peroxidase complex for 15 minutes. Peroxidase activity was visualized using 3-amino-9-ethylcarbazole (AEC, red color) or 3,3’diaminobenzidine (DAB, brown color) with 0.01% H2O2 as substrate. The reaction was terminated by rinsing the sections with distilled water. Counterstaining was performed with hematoxylin.

Data Analysis

For each antibody, three masked sections taken from a standardized sample, encompassing the midperipheral to the central part of the retina of each donor, were examined by two independent observers. When the observers were not in agreement, the section was scored

| Table 1. Association of VEGFR-2 and PAL-E Expression in Microvessels in Retinas of Control, and Diabetic Donor Eyes |

<table>
<thead>
<tr>
<th>Group</th>
<th>PAL-E Staining Distribution*</th>
<th>VEGFR-2 Staining (n)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent</td>
<td>Sporadic</td>
</tr>
<tr>
<td>Control donors</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>Diabetic donors</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>5</td>
</tr>
</tbody>
</table>

For the control group, γ = 0.756, P = 0.063; for the diabetic group, γ = 0.834, P < 0.0001; for the two groups combined, γ = 0.850, P < 0.0001.

* Staining absent; sporadic, less than three positive microvessels per 5-mm section; patchy, localized areas with staining microvessels, but less than 50% of vessels positive; uniform, more than 50% of vessels positive.

| Table 2. Association of VEGFR-3 and PAL-E Expression in Microvessels in Retinas of Control and Diabetic Donor Eyes |

<table>
<thead>
<tr>
<th>Group</th>
<th>PAL-E Staining Distribution*</th>
<th>VEGFR-3 Staining (n)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent</td>
<td>Sporadic</td>
</tr>
<tr>
<td>Control donors</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Diabetic donors</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>5</td>
</tr>
</tbody>
</table>

For the control group, γ = 0.625, P = 0.445; for the diabetic group, γ = 0.190, P = 0.460; for the two groups combined, γ = 0.463, P = 0.035.

* See Table 1 for description of grading scale.
The error of PAL-E grade 2 staining when VEGFR staining is assigned to grade 3 staining when VEGFR staining is assigned grade 2, versus the gold standard for both parameters, the same weight to the error of misclassification. This test provides, in the absence of a perfect classifier, a statistical measure of the correlation between the two variables (PAL-E staining and VEGFR staining), we used the conventional nonparametric rank order \( \tau \)-test, the outcome of which can be interpreted as the correlation coefficient (SPSS ver. 8.0; section Crosstabs; SPSS, Chicago, IL). This test provides, in the absence of a perfect classifier, a statistical measure of the correlation between the two variables.

For statistical analysis of the association between ordinal variables (PAL-E staining and VEGFR staining), we used the conventional nonparametric rank order \( \tau \)-test, the outcome of which can be interpreted as the correlation coefficient (SPSS ver. 8.0; section Crosstabs; SPSS, Chicago, IL). This test provides, in the absence of a perfect classifier, a statistical measure of the correlation between the two variables.

<table>
<thead>
<tr>
<th>Group</th>
<th>VEGFR-2 Staining Distribution*</th>
<th>VEGFR-3 Staining (( \alpha ))†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent</td>
<td>Sporadic</td>
</tr>
<tr>
<td>Control donors</td>
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<td></td>
</tr>
<tr>
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<tr>
<td>Sporadic</td>
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<tr>
<td>Patchy</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Uniform</td>
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<td></td>
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<tr>
<td>Total</td>
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<td>6</td>
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<tr>
<td>Diabetic donors</td>
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<td>1</td>
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<td>Sporadic</td>
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<td>2</td>
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<tr>
<td>Patchy</td>
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</tr>
<tr>
<td>Uniform</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>5</td>
</tr>
</tbody>
</table>

For the diabetic group, \( \gamma = 0.755, P < 0.0001 \); for the two groups combined, \( \gamma = 0.696, P = 0.003 \).

See Table 1 for description of grading scale.

For the diabetic group, \( \gamma = 0.755, P < 0.0001 \); for the two groups combined, \( \gamma = 0.696, P = 0.003 \).

**RESULTS**

**VEGFR Expression in Human Retinas**

**VEGFR-1.** VEGFR-1 was expressed in all microvascular structures that were positive for CD31 in retinas of both control and diabetic eyes (Figs. 1A, 1B, 2D). Staining was more intense in retinal microvessels of diabetic eyes, which often displayed a hypertrophic morphology (Figs. 2C, 2D). In all retinas, granular VEGFR-1 staining was also observed outside the retinal vasculature, in the inner limiting membrane, the ganglion cell layer, the inner plexiform and nuclear layer, and the outer plexiform layer (Fig. 1A).

**VEGFR-2.** Staining of VEGFR-2 was absent in retinal microvessels in 13 of 18 control eyes (Fig. 1C). A few weakly stained microvessels showed a sporadic distribution pattern in 4 of 18 control eyes, and a patchy distribution pattern was observed in one control eye. In diabetic eyes, staining of VEGFR-2 was observed in microvessels in the ganglion cell layer and inner nuclear layer in 18 of 27 retinas. Staining was sporadic in 5 cases, patchy in 12 cases, and uniform in 1 case (Table 1). Staining of VEGFR-2 in retinal microvessels was weak but distinct and displayed a fine granular pattern (Fig. 2E).

Qualitative and semiquantitative analyses showed that vascular staining of VEGFR-2 was associated with PAL-E staining (Figs. 2A, 2B; Table 1). In diabetic eyes, PAL-E-positive microvessels were larger than control microvessels. When present, microaneurysms were also positive for VEGFR-2 and PAL-E (data not shown).

In all eyes, variable weak granular staining for VEGFR-2 was observed outside the retinal vasculature in neural and glial elements of the ganglion cell layer, the inner plexiform and nuclear layer, the outer plexiform and nuclear layers (Figs. 1C, 2E), and occasionally in the outer limiting membrane (data not shown).

**VEGFR-3.** In control eyes, retinal vascular staining of VEGFR-3 was absent (16/18 cases; Fig. 1E, Table 2) or sporadic (2/18 cases), whereas VEGFR-3 staining was observed in microvessels of retinas of 11 of 27 diabetic eyes (Table 2). VEGFR-3 was expressed in microvessels of the inner nuclear layer and the ganglion cell layer (Fig. 2G). VEGFR-3 staining was mainly found in retinas with distinct PAL-E expression (Table 2), although a number of PAL-E-positive cases did not show any vascular staining of VEGFR-3. In addition, the association between distribution patterns of VEGFR-3 and -2 staining were high (Table 3).

In all eyes, staining of VEGFR-3 was also observed outside the retinal vasculature. It was present in neural elements of the inner plexiform layer, the inner nuclear layer, and the inner part of the outer plexiform layer (Figs. 1E, 2G), sometimes in a pearl-necklace-like configuration (Fig. 1E).

**VEGFR-Induced Retinopathy in Monkeys**

Changes in monkey eyes due to PBS and VEGF injection have been described.38 Briefly, in both VEGF-injected eyes, retinal venous dilation was observed by funduscopic evaluation of the eyes on day 9. The eyes were without biomicroscopic signs of inflammation, and intraocular pressures remained at least 25 mm Hg at all time points. Hematoxylin-eosin staining of sections of the central retina of the eyes injected with VEGF demonstrated edematous changes.

CD31 staining revealed a normal distribution pattern of microvessels throughout the retinas of both PBS- and VEGF-injected eyes. However, staining of CD31 was stronger in the VEGF-injected eyes (Figs. 3C, 3D). The vessels in these eyes were hypertrophic, more dilated, and slightly more tortuous than in PBS-injected eyes. Staining of PAL-E was not present in retinas of PBS-injected eyes, whereas it was uniformly distributed in retinas of VEGF-injected eyes (data not shown).

**VEGFR Expression in Monkey Retinas**

**VEGFR-1.** In PBS- and VEGF-injected eyes, staining of VEGFR-1 was observed inside and outside the retinal vasculature, which was consistent with findings in human eyes (Figs. 3E, 3F). Staining intensity was higher in retinal vessels of VEGF-injected eyes than in PBS-injected eyes. Variable diffuse staining was present outside the retinal vasculature.
in neural or glial elements of the ganglion cell layer, the inner plexiform and nuclear layers, and the outer plexiform layer (Figs. 3E, 3F).

VEGFR-2. In PBS-injected eyes, retinal microvessels were negative for VEGFR-2 (Fig. 3G), whereas retinal microvascular staining of VEGFR-2 was localized in a granular pattern in...
VEGF-injected eyes (Fig. 3H). VEGFR-2-positive microvessels were mainly localized in the ganglion and nerve fiber layers, whereas most microvessels in the inner nuclear layer were negative (Fig. 3H).

Variable weak granular staining of VEGFR-2 was observed in all monkey eyes outside the vasculature in neural or glial elements of the ganglion cell layer, the inner plexiform and nuclear layers, and the outer plexiform layer (Figs. 3G, 3H), which was consistent with findings in human eyes.

**VEGFR-3.** Retinal microvascular staining of VEGFR-3 was observed in the ganglion and nerve fiber layers, whereas microvessels in the inner nuclear layer were negative in VEGF-injected eyes (Fig. 3J). In eyes injected with PBS, staining of VEGFR-3 was absent in the retinal microvessels (Fig. 3I).

In all eyes, staining of VEGFR-3 was observed outside the retinal vasculature in neural elements of the inner plexiform layer and the inner part of the outer plexiform layer in a pearl-necklace-like configuration (Fig. 3I, 3J).

**DISCUSSION**

On the basis of the present study, we conclude that in control and diabetic human eyes and PBS- and VEGF-injected monkey eyes, all three VEGFRs are expressed in neural elements of the retina outside the microvasculature; in control human and PBS-injected monkey eyes, VEGFR-1 is expressed in the microvascular wall, whereas VEGFR-2 and -3 are generally absent; in diabetic eyes, leaky microvessels with PAL-E expression also express VEGFR-2; in a subset of retinal PAL-E-positive microvessels of diabetic eyes, VEGFR-3 is expressed as well; and in VEGF-A-induced retinopathy in monkey eyes, VEGFR-2 and -3 expression is upregulated in retinal microvessels.

We have studied human donor eyes without information on the type of diabetes mellitus or the presence and/or degree of DR, for reasons of privacy. However, we were able to investigate the relation between VEGFR expression and vascular leakage by staining adjacent sections for the endothelial antigen PAL-E, a specific marker for capillary leakage in diabetic retina. Although PAL-E cannot be regarded as a direct marker for DR, microvascular leakage is associated clinically (and thus histologically) with other features of DR.

VEGF is a major cytokine that causes vascular leakage and angiogenesis, and its expression has been demonstrated in ischemic retina, in line with the clinical association of retinal ischemia with retinal leakage and ocular angiogenesis. However, in situ hybridization and immunohistochemistry with polyclonal antibodies have shown mRNA and protein expression of VEGF-A and VEGFR-1, -2, and -3 also in the normal retina, where vascular leakage and angiogenesis do not occur. Our present data help to explain these expression patterns. By the use of highly specific monoclonal antibodies, we found all three VEGFrs to be expressed in nonvascular cells in retinas of human control eyes and PBS-treated monkey eyes, suggesting that VEGFs have a physiologic function in retinas with respect to neural elements, which is in agreement with the neurotrophic function of VEGF-A. Only VEGF-1, a receptor that mediates migration but not permeability or mitosis, was ubiquitously localized in microvascular walls in retinas. VEGFR-1 is expressed by retinal pericytes in vitro. VEGFR-1 expression by pericytes in vivo may corroborate our observations of VEGFR-1 expression in the walls of retinal microvessels, where it may enable pericytes rather than endothelial cells to respond initially to VEGF signaling under quiescent conditions. Alternatively, the vascular staining pattern of VEGFR-1 may represent a localization of the soluble form of this receptor in the basement membrane of retinal microvessels. This possibility is supported by our observation of VEGFR-1 staining in the inner limiting membrane (i.e., the basement membrane of Müller cells), which indicates a localization of VEGFR-1 in the extracellular matrix, rather than on cells. sVEGFR-1 has been suggested to act as a scavenger for VEGF-A, thereby protecting vessels from becoming leaky or proliferative at low levels of VEGF-A. Ultrastructural studies are needed to further investigate these interesting possibilities, in that our light microscopic analysis did not allow an exact localization of VEGFR-1 in endothelial cells, pericytes, and/or extracellular matrix elements of microvessels.

Sporadic PAL-E expression with weak staining intensity was found in a number of control retinas, as previously described. Sporadic VEGFR-2 and -3 expression with weak staining intensity was found in a few control eyes as well (5/18 and 2/18 eyes, respectively), in some cases associated with sporadic PAL-E expression (3/18 and 1/18 eyes, respectively). The functional relevance of this finding in control eyes remains to be elucidated, but a striking phenotypic shift of expression of PAL-E and VEGFRs was observed in diabetic eyes. In these human eyes and in two monkey eyes treated with a prolonged regimen of VEGF-A, we observed microvascular expression of VEGFR-1, -2, and -3. Microvascular expression of VEGFR-2 was associated with leaky vessels in the diabetic retina, as can be concluded from the significant association with PAL-E expression. The observations in monkey eyes injected with VEGF-A indicate that high levels of VEGF-A and/or prolonged exposure to VEGF induces VEGFR-2 expression in retinal vascular cells. In brain capillaries, a similar mechanism of VEGFR-2 induction has been suggested. This may be a direct effect of VEGF-A on endothelial cells through their constitutive expression of VEGFR-1, a very low-level baseline expression of VEGFR-2 that remained undetectable by immunohistochemistry, or an indirect effect of VEGF-A through another route that induces a secondary signal acting on endothelial cells.

In light of our previous demonstration of a relation between PAL-E expression and established vascular leakage in DR and together with our current observations, the following hypothetical scenario in the course of DR may be proposed: Retinal ischemia causes overexpression of VEGF-A, which triggers expression of VEGFR-2 on microvascular cells, possibly through VEGFR-1, leading to vascular leakage (PAL-E expression) and eventually to angiogenesis caused by VEGF-A’s signaling this receptor. Our detailed observations in human diabetic eyes support this scenario. We found VEGFR-2 expression mainly in retinal areas with microvascular leakage (suggesting the presence of established DR), as recognized by vascular staining of the PAL-E antigen. It has been demonstrated that VEGF-A is overexpressed in such areas. In addition, expression of VEGFR-3 correlated well with expression of VEGFR-2 in diabetic eyes, suggesting induction of VEGFR-2 and -3 and a role for their respective ligands in such areas.

In 9 of 27 diabetic eyes (Table 2) and in monkey eyes injected with VEGF-A, we observed VEGFR-3 upregulation associated with increased PAL-E staining in retinal microvessels. In diabetic eyes, VEGFR-3 staining was observed in vessels in the deeper layers of the retina, in which VEGF-A is overexpressed in DR. In the monkey retina, VEGFR-3 expression was localized in vessels of the inner layers of the retina, suggesting a dose–response effect of exogenous VEGF-A, as is the case with VEGFR-2. VEGF-A is not a ligand for VEGFR-3, and VEGFR-3 and its ligand VEGF-C were previously considered to be involved in lymphangiogenesis in the adult, and in angiogenesis in embryos and tumors. Our findings indicate distinct and differing roles of VEGFR-3 and its ligands VEGF-C and/or -D in the normal and diabetic retina, and our studies in monkey eyes suggest that its expression is induced by VEGF-A. Outside the vasculature, VEGFR-3 staining was...
observed unexpectedly in a distinct pearl-necklace–like configuration in the outer plexiform layer in both control and diabetic retinas, a pattern highly suggestive of staining of synaptic complexes of cone photoreceptors.

Finally, it has been suggested that VEGF-A upregulation also plays a role in preclinical human DR and in early retinopathy in rat models of streptozotocin-induced diabetes and that preclinical increased vascular expression of VEGFR-2 mediates this role. In our study, we did not find evidence for such early expression, in that diabetic eyes without PAME staining did not differ from the non-diabetic control eyes in distribution patterns of VEGFR staining. The discrepancy between studies in rat models of experimental DR and our study may exist because small rodents with experimental diabetes do not represent a good model of human preclinical DR, and/or because vascular expression of VEGFR-2 in control eyes and preclinical DR is below the detection level of our immunohistochemical method.

In summary, our findings indicate a dual role of VEGFs in the physiology and pathophysiology of the retina, and suggest that VEGFR-2 and -3 signaling of vascular cells occurs relatively late in the diabetic retina, in areas with established microvascular leakage associated with DR, possibly initiated by high levels of VEGF-A produced in these areas.

Acknowledgments

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