Vascular Endothelial Growth Factor and Vascular Permeability Changes in Human Diabetic Retinopathy

Michaela Kunz Mathews, Carol Merges, D. Scott McLeod, and Gerald A. Lutty

Purpose. The authors used histochemical analysis to determine whether increased vascular endothelial growth factor (VEGF) immunoreactivity in diabetic retinal vessels is related to increased vascular permeability, as indicated by human serum albumin (HSA) immunostaining, or to presumed retinal hypoxia, as demonstrated by decreased vascularity. A correlation between VEGF and HSA in cryosections with angiopathic changes in the adenosine diphosphatase (ADPase) flat-embedded fellow retinas was sought. Because VEGF is a heparin-binding protein, the relation between VEGF and heparan sulfate proteoglycan (HSPG) immunoreactivities was also investigated.

Methods. Cryopreserved eyes from 18 diabetic and 9 nondiabetic subjects removed after death were sectioned and immunohistochemical analysis was performed with antibodies against VEGF, HSA, HSPG, vWF (von Willebrand factor), and collagen IV. The fellow retinas were prepared by our ADPase flat-embedding technique to determine the degree of diabetic retinopathy. The number of positive vessels for each antibody and antibody localizations were determined by light microscopy.

Results. The average number of VEGF-stained vessels in diabetic retinas was significantly higher than in nondiabetic retinas \( (P = 0.04) \). In diabetic retinas, there was a positive correlation between the distribution of VEGF-positive vessels and the distribution of HSA- and HSPG-positive vessels. No such correlation was observed in nondiabetic eyes. In many cases, HSPG immunoreactivity appeared colocalized with VEGF immunoreactivity, suggesting VEGF binding to HSPG. The comparison with the ADPase flat-embedded fellow retinas suggested that increased VEGF immunoreactivity and vascular permeability may occur before morphologic changes in the vasculature.

Conclusions. Vascular endothelial growth factor immunoreactivity was correlated with increased vascular permeability to macromolecules and appears to be increased in diabetic subjects before the onset of retinopathy. Invest Ophthalmol Vis Sci. 1997;38:2729-2741.

The retina is one of the most metabolically active tissues in the body. Deprivation of oxygen, especially chronic hypoxia as occurs in systemic diseases such as diabetes or sickle cell disease, causes functional damage. A repair mechanism some organs provide to prevent tissue damage in cases of compromised circulation is growth of new vessels (angiogenesis). In the retina, however, this repair mechanism is often more harmful than beneficial. New blood vessels can grow past the limits of the retina onto the retinal surface and often leak or break, causing hemorrhage, scarring, and retinal detachment, which may lead to severe visual loss or blindness.

In 1948, Michaelson\(^1\) hypothesized that a diffusible factor, “factor X,” was produced by ischemic retina and induced the growth of new vessels. A recently identified substance that appears to have characteristics similar to Michaelson’s theoretical “factor X” is vascular endothelial growth factor (VEGF), also called vascular permeability factor. Vascular endothelial growth factor is a 46-kD dimeric glycoprotein with partial structural homology with platelet-derived growth factor.\(^2\) Four isoforms can be produced by alternative splicing of messenger RNA, 121, 165, 189, and 206 amino acid residues long.\(^3\) The two shorter

---


VEGF isoforms are secreted; the two longer isoforms appear to be cell-associated.\(^1\) VEGF binds with high affinity to specific tyrosine kinase receptors on endothelial cells—Flt-1, Flk-1 (KDR), and Flt-4.\(^2\) Binding of VEGF to the cell surface is potentiated by heparin,\(^5\) and VEGF binds to heparan sulfate proteoglycan (HSPG) and heparin, a strategy exploited for its purification.\(^7\)

In the retina, VEGF may function as a paracrine or autocrine growth factor because its production has been observed in vitro by retinal endothelial cells as well as by pericytes and retinal pigment epithelial cells.\(^8\)\(^-\)\(^11\) A crucial characteristic of VEGF production, whether paracrine or autocrine, is that its expression can be induced by hypoxia.\(^15\) This has been demonstrated in vitro using retinal endothelial cells, pericytes,\(^9\) and retinal pigment epithelial cells\(^11\) and in vivo in hypoxic areas of human retina.\(^16\) In a primate model for retinal hypoxia, VEGF messenger RNA was detected in retina and VEGF protein was detected in intraocular fluids at appropriate times in relation to induction of hypoxia.\(^15,16\) The association of VEGF production with hypoxia has also been demonstrated in several animal models during presumed hypoxia of retinal development and after the vasoobliterative stage in oxygen-induced retinopathy.\(^17,18\)

The biologic effects of VEGF can induce the angiopathic processes that are hallmarks of diabetic retinopathy: increased vascular permeability and angiogenesis. Vascular endothelial growth factor promotes the proliferation and the migration of retinal endothelial cells in vitro\(^10\) and stimulates angiogenesis in the chorioallantoic membrane assay\(^5\) and in the corneal neovascularization assay.\(^19\) Vascular endothelial growth factor induction of increased vascular permeability has been demonstrated in diabetic rat retinal vessels,\(^20\) in tumors,\(^21,22\) and in delayed hypersensitivity skin reactions.\(^23\)

The involvement of VEGF in human diabetic retinopathy has been suggested recently. We have observed increased VEGF immunoreactivity in blood vessels of diabetic retinas compared to nondiabetic control retinas.\(^24\) Pe’er et al\(^14\) have observed increased VEGF messenger RNA in inner retina of subjects with diabetes. Vitreous VEGF levels are increased in eyes with proliferative diabetic retinopathy,\(^25,26\) and VEGF messenger RNA has been demonstrated in diabetic neovascular membranes.\(^27\)

The purpose of this study was to gain further insights into the role of VEGF in diabetic retinopathy. We used our adenosine diphosphatase (ADPase) flat-embedded technique\(^28\) to analyze the retinal vasculature in one eye and immunohistochemical analysis\(^29,30\) of the fellow eye from each subject to determine if there was a correlation between VEGF immunostaining and diabetes-related angiopathic changes. We performed semiquantitative morphometric analysis of VEGF and human serum albumin (HSA) immunoreactivities in diabetic and nondiabetic retinas to determine if there was a correlation between the distribution of VEGF-positive vessels and increased vascular permeability. We also attempted to correlate VEGF immunoreactivity with loss in viable vasculature and, therefore, relate it to tissue hypoxia. Because VEGF is a heparin-binding growth factor and its action appears to be heparin-dependent, we investigated the localization of HSPG in retinas of diabetic and nondiabetic subjects and correlated it with VEGF immunoreactivity.

**MATERIALS AND METHODS**

Human eyes removed after death were obtained from National Disease Research Interchange (Philadelphia, PA) and Medical Eye Bank of Maryland (Baltimore, MD). Eyes from 18 diabetic and 9 nondiabetic subjects were examined. The mean age of donors was 69 years (range, 50 to 91) in the diabetic group and 76 years (range, 44 to 92) in the nondiabetic group. The mean death-to-enucleation time was 3.3 hours (range, 1 to 8 hours) for diabetic subjects and 4.1 hours for nondiabetic subjects (range, 1 to 13 hours). The mean postmortem time was 24.5 hours (range, 18 to 34 hours) for diabetic subjects and 25.9 hours (range, 12 to 42 hours) for nondiabetic subjects. There was no significant difference between the diabetic and nondiabetic group in age \((P = 0.24)\), death-to-enucleation time \((P = 0.55)\), or postmortem time \((P = 0.57)\) in the diabetic group, 3 patients were diagnosed with insulin-dependent diabetes mellitus type 1, 11 patients with insulin-dependent diabetes mellitus type 2, and 4 patients with non-insulin-dependent diabetes mellitus type 2 (Table 1). In 16 of 18 diabetic eyes and 6 of 9 nondiabetic eyes, serial sections for immunohistochemistry were obtained from nasal retina. All sections included retina from the peripapillary area to ora serrata except the temporal blocks, which included retina from macula to ora serrata.

Immunohistochemical analysis was performed on serial 8-μm sections of cryopreserved tissue as previously described.\(^29,30\) Before embedding and freezing the tissue, gross examination of all tissue was performed and gross photographs were taken. The sections were incubated in primary antibodies for 20 hours at 4°C, with the antibodies applied in the following order to serial sections: mouse anti-collagen IV ascites diluted 1:60,000; Chemicon International (Temecula, CA); rabbit anti-vWF (DAKO, Carpinteria, CA) at 1.16 μg/ml; rabbit anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) at 0.025 μg/ml; mouse anti-HSPG at 0.4 μg/ml (Chemicon); and rabbit antiserum against HSA (Nordic Immunology, Tielburg, The Netherlands) diluted 1:500,000. The series was repeated so that each primary antibody was applied to duplicate slides (two sections per slide) in
TABLE 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Case Number</th>
<th>Location</th>
<th>Age (years)</th>
<th>PMT (hours)</th>
<th>DET (hours)</th>
<th>Cause of Death</th>
<th>History</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic patients</td>
<td>Superior nasal</td>
<td>51</td>
<td>30</td>
<td>6</td>
<td>Cardiac arrest</td>
<td>IDDM (7 years)</td>
</tr>
<tr>
<td>2</td>
<td>Nasal</td>
<td>57</td>
<td>17</td>
<td>3</td>
<td>Metastatic breast cancer</td>
<td>IDDM</td>
</tr>
<tr>
<td>3</td>
<td>Nasal</td>
<td>57</td>
<td>35</td>
<td>2.5</td>
<td>Pulmonary edema, hyperglycemia</td>
<td>IDDM (1 year)</td>
</tr>
<tr>
<td>4</td>
<td>Nasal</td>
<td>58</td>
<td>23</td>
<td>2</td>
<td>Cardiac arrest, possible MI</td>
<td>IDDM (26 years ID), HTN</td>
</tr>
<tr>
<td>5</td>
<td>Superior-nasal</td>
<td>58</td>
<td>19.5</td>
<td>2</td>
<td>Multisystem failure</td>
<td>IDDM (12 years)</td>
</tr>
<tr>
<td>6</td>
<td>Nasal</td>
<td>64</td>
<td>30</td>
<td>3</td>
<td>Chronic heart failure</td>
<td>IDDM; HTN</td>
</tr>
<tr>
<td>7</td>
<td>Nasal</td>
<td>68</td>
<td>21.5</td>
<td>1</td>
<td>Stroke</td>
<td>IDDM (12 years ID)</td>
</tr>
<tr>
<td>8</td>
<td>Nasal</td>
<td>69</td>
<td>17</td>
<td>6</td>
<td>Cardiac arrest, renal failure</td>
<td>IDDM</td>
</tr>
<tr>
<td>9</td>
<td>Nasal</td>
<td>71</td>
<td>39</td>
<td>3</td>
<td>Cardiopulmonary arrest, MI</td>
<td>IDDM (30 years ID)</td>
</tr>
<tr>
<td>10</td>
<td>Nasal</td>
<td>74</td>
<td>26</td>
<td>3</td>
<td>Renal failure</td>
<td>NIDDM, Parkinson, HTN</td>
</tr>
<tr>
<td>11</td>
<td>Superior-temporal</td>
<td>75</td>
<td>24</td>
<td>2.5</td>
<td>Chronic heart failure</td>
<td>IDDM (10 years ID)</td>
</tr>
<tr>
<td>12</td>
<td>Nasal</td>
<td>76</td>
<td>21</td>
<td>8</td>
<td>Cerebral hemorrhage</td>
<td>NIDDM</td>
</tr>
<tr>
<td>13</td>
<td>Nasal</td>
<td>79</td>
<td>42</td>
<td>2</td>
<td>Cardiopulmonary arrest, bladdar cancer</td>
<td>IDDM 1</td>
</tr>
<tr>
<td>14</td>
<td>Nasal</td>
<td>80</td>
<td>21</td>
<td>1</td>
<td>Possible MI</td>
<td>NIDDM</td>
</tr>
<tr>
<td>15</td>
<td>Nasal</td>
<td>81</td>
<td>13</td>
<td>3.5</td>
<td>MI</td>
<td>IDDM (16 years)</td>
</tr>
<tr>
<td>16</td>
<td>Nasal</td>
<td>83</td>
<td>12</td>
<td>3</td>
<td>Prostate cancer</td>
<td>IDDM, HTN</td>
</tr>
<tr>
<td>17</td>
<td>Nasal</td>
<td>91</td>
<td>23</td>
<td>5</td>
<td>Cardiac arrest</td>
<td>NIDDM, CAD, HTN</td>
</tr>
<tr>
<td>18</td>
<td>Nasal</td>
<td>44</td>
<td>34</td>
<td>13</td>
<td>Cardiopulmonary arrest</td>
<td>Pneumocystic infection</td>
</tr>
<tr>
<td>19</td>
<td>Nasal</td>
<td>68</td>
<td>26</td>
<td>2</td>
<td>Cardiac arrest</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Nasal</td>
<td>68</td>
<td>38</td>
<td>4</td>
<td>Cardiac arrest</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Nasal</td>
<td>72</td>
<td>23.5</td>
<td>3</td>
<td>Cardiac arrest</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Temporal</td>
<td>79</td>
<td>26</td>
<td>4</td>
<td>Respiratory failure</td>
<td>Ischemic heart disease</td>
</tr>
<tr>
<td>23</td>
<td>Nasal</td>
<td>84</td>
<td>29</td>
<td>2</td>
<td>Metastatic pancreatic cancer</td>
<td>COPD</td>
</tr>
<tr>
<td>24</td>
<td>Nasal</td>
<td>88</td>
<td>27</td>
<td>2</td>
<td>Cardiac arrest</td>
<td>HTN</td>
</tr>
<tr>
<td>25</td>
<td>Nasal</td>
<td>90</td>
<td>24</td>
<td>1</td>
<td>Cardiac arrest</td>
<td>CHF, HTN</td>
</tr>
<tr>
<td>26</td>
<td>Nasal</td>
<td>92</td>
<td>25.5</td>
<td>6</td>
<td>CAD</td>
<td>CAD</td>
</tr>
<tr>
<td>27</td>
<td>Nasal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PMT = postmortem time; DET = death-enucleation time; IDDM 1 = insulin-dependent diabetes mellitus type 1; IDDM 2 = insulin-dependent diabetes mellitus type 2; NIDDM = non-insulin-dependent diabetes mellitus; HTN = hypertension; CHF = congestive heart failure; COPD = chronic obstructive pulmonary disease; MI = myocardial infarction; CAD = coronary artery disease.

each experiment. Biotinylated second-step antibodies (diluted 1:500) were applied for 30 minutes followed by streptavidin peroxidase (diluted 1:500) and incubated for 45 minutes at room temperature (both second- and third-step reagents from Kirkegaard and Perry Laboratories, Gaithersburg, MD). Peroxidase was developed with aminoethylcarbazol, resulting in a red reaction product. One of the two sections on each slide was counterstained with hematoxylin.

Control sections were incubated with nonimmune IgG (Jackson ImmunoResearch, West Grove, PA) at the same protein concentration as the primary antibody. To demonstrate that VEGF antibody binding was bona fide, we performed blocking experiments on sections from five subjects (subjects 7, 8, 14, 22, and 24) as follows. Vascular endothelial growth factor antibody diluted with bovine serum albumin (0.025 µg antibody/ml solution) and VEGF antibody with the VEGF peptide used as antigen (VEGF peptide concentrations of 0.25 µg/ml and 0.5 µg/ml) were incubated at 4°C overnight and centrifuged before immunohistochemistry.

Semiquantitative analysis was performed on non-counterstained sections using a Zeiss light microscope (Carl Zeiss, Oberkochen, Germany) with a 16× objective. The number of stained vessels for each antibody was counted sequentially, field by field, from posterior pole to periphery in each section. The average number of stained vessels per visual field on the microscope (1.3 mm²) ± standard deviation was then determined. We also compared the distribution of positive vessels in the same fields from central to peripheral retina for each antibody in each section. For qualitative analysis, bookkeeping of fields analyzed, and determination of colocalization of two antibodies in the same vessels, we captured images of corresponding fields of retina stained with different antibodies, using our image analysis system. The system consisted of a Zeiss Photomicroscope II, a Hamamatsu videocamera (model #C2400-77; Hamamatsu City, Japan), and the
The number of immunohistochemical positive vessels on sections and the extent of angiopathic changes in the whole retina from the fellow eye were compared using the ADPase flat-embedded fellow retinas of the same subjects. Angiopathy in the ADPase retinal preparations was scored according to the retinopathy severity scale developed by the Early Treatment Diabetic Retinopathy Study Research Group. Statistical evaluation of the data involved calculating probability values using the Student's t-test for two samples assuming unequal variances and linear regression analysis of the distribution curves, thus obtaining curve equations and r² values.

RESULTS

Vascular endothelial growth factor immunoreactivity was observed most prominently in the walls of retinal vessels of all sizes (Fig. 1A). Specificity of the VEGF antibody was demonstrated by preincubating the antibody with peptide used as antigen before performing immunohistochemistry. Preincubation of antibody with blocking peptide resulted in only minimal VEGF staining in retina—in other words, these sections were comparable to control sections incubated with nonimmune IgG (Fig. 1B). The vascular sites of VEGF labeling were similar in retinas from diabetic and nondiabetic subjects but there were significantly more VEGF-positive vessels and a greater staining intensity in diabetic eyes (Figs. 2C and 2D). In retinal vessels of diabetic eyes, mostly uniform staining of the luminal surface and the vessel wall occurred; in nondiabetic eyes, however, the staining pattern was generally weak and patchy—only part of the vessel wall was stained. In addition to vessel wall staining, distinct perivascular immunoreactivity and weak diffuse staining of the nuclear layers were observed in some diabetic eyes (see Fig. 2C). Perivascular and nuclear layer staining was
very weak in most nondiabetic eyes (see Fig. 2D). Some leukocytes and the internal limiting membrane were also positive for VEGF. No staining of photoreceptors, ganglion cells, or Müller cell processes was observed in any of the eyes examined.

The average number of VEGF-stained vessels in diabetic eyes was 47.5% higher than in nondiabetic eyes (Fig. 3, P = 0.04). The distribution of VEGF-positive vessels was different in diabetic retinas compared to nondiabetic ones (Fig. 4). In nondiabetic eyes, we found fewer VEGF-positive vessels in central retina (posterior pole) than in equatorial and periph-
FIGURE 3. The average number of vessels positive for vascular endothelial growth factor in diabetic retinas was significantly higher than in nondiabetic retinas \((P = 0.04)\). Because all vessels with human serum albumin (HSA) immunoreactivity were considered in this analysis, disregarding the intensity of immunoreactivity, there was no statistically significant increase in average numbers of HSA-positive vessels in diabetic eyes when total vessel numbers per section were considered.

eral regions, even though the number of viable vessels (vWF-positive) was highest in central retina. Vessels positive for VWF reflected the normal anatomic pattern of the retinal vasculature in both sets of eyes—in other words, there were fewer vessels in the periphery in nondiabetic but fewer viable vessels in all areas of the diabetic retina (see Fig. 4). The highest number of VEGF-positive vessels in diabetic eyes, however, was located in the central retina, with a steady decrease toward the periphery, paralleling the distribution of presumed viable vessels that were positive for VWF. For diabetic eyes, when VEGF-positive vessels in fields 1 to 3 were compared with fields 7 to 10, there were significantly more positive vessels in the central retina \((P = 0.0009)\). The opposite was true for nondiabetic eyes, in which there were more VEGF-positive vessels in the peripheral retina (fields 7 to 10) than in the central retina (fields 1 to 3; \(P = 0.015\)). Far peripheral field 11 was not used in this comparison because it was not present in blocks from all eyes. The number of VEGF-positive vessels in nondiabetic retinas never exceeded the number of VEGF-positive vessels in diabetic retinas in all central and equatorial fields (fields 1 to 6; see Fig. 4), but the distinction was not clear in fields 7 to 10. When each field was compared for the two groups, there was a significant difference \((P < 0.05)\) between diabetic and nondiabetic eyes in fields 1 to 6 but not in fields 7 to 10 (asterisks in Fig. 4).

Human serum albumin staining was also observed in retinas from both diabetic and nondiabetic subjects (Figs. 2E and 2F). In nondiabetic eyes, staining was weak and mostly limited to some vessel walls and some areas of the inner photoreceptor matrix. In diabetic eyes, HSA staining was much more intense and was observed in vessel walls, diffusely around blood vessels, in the inner photoreceptor matrix, at the internal limiting membrane, and in vitreous (see Fig. 2E). Human serum albumin immunoreactivity patterns and intensities were very different between diabetic and nondiabetics subjects, and the number of HSA-positive vessels (even if weakly labeled in nondiabetic subjects) was slightly higher in diabetic subjects, although there were fewer VWF-positive vessels in diabetic subjects. When all HSA-positive vessels were included, however, the difference between the two groups was not statistically significant (see Fig. 3). When we conducted linear regression analysis comparing average numbers of VEGF-positive and HSA-positive blood vessels with respect to their geographic distribution in retina, we established a good correlation for diabetic eyes \((r^2 = 0.44, P = 0.049)\). In nondiabetic retinas, the correlation between HSA- and VEGF-immunoreactive vessels...
Immunohistochemical Analysis of VEGF in Human Diabetic Retina

The average vessel numbers per visual field on the light microscope (1.3 mm²) were plotted from central retina (field 1, F1) to peripheral retina (F11). The number of viable vessels in diabetic eyes (solid squares) was compared with nondiabetic eyes (open squares) in all fields. The number of VEGF-positive vessels in diabetic eyes (solid circles) was compared with nondiabetic eyes (open circles). The asterisks indicate data points (fields) in which there was a statistically significant difference (P < 0.05) between the number of VEGF-positive vessels in diabetic versus nondiabetic retinas.

Assuming that nonperfusion in diabetic eyes results in retinal hypoxia, we tried to determine the relation between nonperfusion—hypoxia and VEGF immunoreactivity. Serial sections were incubated with antibodies against collagen IV (labels both viable blood vessels and “ghost vessels” [vessels without endothelial cells]), vWF (a marker for vessels with viable endothelial cells), and VEGF (Fig. 6). We hypothesized that nonperfused areas of retina would have collagen IV-positive vessels that were vWF-negative because ghost vessels lack endothelial cells, the cells that produce vWF. Therefore, the number of ghost vessels could be calculated by subtracting the number of vWF-positive vessels (viable vessels) from the number of collagen IV-positive vessels. The resulting numbers of ghost vessels were very small, and no statistically significant difference in the number of ghost vessels was observed between diabetic and control retinas, suggesting minimal nonperfusion in the areas analyzed.

We also compared the average number of VEGF-stained vessels with the severity of angiopathic changes in ADPase-incubated retinas from the fellow eyes. This comparison of fellow eyes seemed reasonable because diabetic retinopathy is a relatively bilateral disease. We realize that there can be substantial differences between eyes, but there also can be variability in the severity of angiopathy in different quadrants of the same eye. Because we could not acquire angiograms of the eyes used in this study, evaluation of angiopathy in fellow eyes was our only possible means of assessment. The ADPase flat-embedding method allowed us to determine the severity of diabetic retinopathy in the fellow eyes and to assess the angiopathic changes with greater accuracy and in greater detail than could be accomplished from fluorescein angiograms.

In diabetic subjects, we distinguished three groups based on the severity of the following angiopathic changes: capillary dropout (reduction in ADPase-positive vessels), aneurysms, and intraretinal vascular abnormalities (IRMA). One subject (#6) had severe nonproliferative retinopathy: extensive capillary dropout and numerous microaneurysms and IRMA formations. Ten subjects (#2, 4, 5, 7–10, 14, 16, 17) had mild to moderate nonproliferative retinopathy: small areas of capillary dropout and a few microaneurysms and IRMA formations (fewer than Early Treatment Diabetic Retinopathy Study Research Group standard photograph 8A). Seven subjects (#1, 3, 11–13, 15, 18) had no retinopathy (no angiopathic changes). None of the retinas incubated for ADPase activity had proliferative retinopathy. In most cases, the numbers of VEGF- and HSA-positive vessels were directly related to the severity of angiopathic changes in the fellow eyes; more severe retinopathy was associated with more VEGF-positive vessels and more pronounced HSA leakage. Nevertheless, this comparison also revealed six cases (33% of the diabetic subjects) in which the severity of retinal dis-
FIGURE 6. Comparison of serial sections from a diabetic (A, C, E; case 17) and a nondiabetic (B, D, F; case 28) retina stained with antibodies against von Willebrand factor (A, B), collagen IV (C, D), and vascular endothelial growth factor (VEGF) (E, F) to demonstrate VEGF immunoreactivity mainly in viable vessels. The arrowhead designates the same vessel in the serial sections from each subject. Magnification, ×115.

ease was not related to increased VEGF immunostaining (Fig. 7). Case 11 (Figs. 7B and 7D) had no angiopathic changes in the flat-embedded fellow retina. Case 5 (Figs. 7A and 7C) had a clinical history of type 1 insulin-dependent diabetes mellitus (26 years’ duration) and severe angiopathic changes (capillary dropout, IRMA, microaneurysms) in the retina from the fellow eye. Both cryopreserved retinas from these subjects, however, showed an equivalent number of VEGF-positive vessels and reaction product intensity, as well as a significant amount of serum albumin staining (case 11 shown in Fig. 2F; case 5 not shown).

To determine the possible role of HSPG as a VEGF binding site, we performed parallel immunohistochemical analysis with VEGF and HSPG antibodies. Immunoreactivity with HSPG was observed in most blood vessel walls and in the internal limiting membranes of diabetic and nondiabetics eyes. In diabetic retinas only, HSPG immunoreactivity in vessel walls was colocalized with VEGF immunoreactivity in many cases (Figs. 8A and 8C). When the distributions of VEGF-positive and HSPG-positive vessels were compared, a good correlation could be established for diabetic eyes ($r^2 = 0.759$) but not for nondiabetic eyes ($r^2 = 0.035$, $P < 0.0001$) (Fig. 9).
**DISCUSSION**

The VEGF localization we observed in this study was comparable in distribution to what we have previously observed, and as before, the prevalence of VEGF immunoreactivity was far greater in diabetic retinas than in nondiabetic retinas. In this study, we determined that the number of VEGF-positive vessels was significantly elevated in diabetic eyes and that the distribution of VEGF-positive vessels in diabetic retina was different from the distribution in the nondiabetic retina. The greatest number of VEGF-positive vessels in diabetic eyes was found in central retina, where most of the angiopathic changes are known to occur in diabetic retinopathy. As the number of viable vessels decreased toward peripheral retina, the number of VEGF-positive vessels also decreased, although there was no area in diabetic retinas with fewer VEGF-positive vessels than in nondiabetic retinas. The number of VEGF-positive vessels in nondiabetic eyes was slightly increased in peripheral retina despite a decrease in the number of viable vessels, which represents the normal anatomic distribution of blood vessels. We attributed this observation in nondiabetic subjects to the presence of degenerative changes in peripheral retina caused by age or other vascular diseases (e.g., hypertension, atherosclerosis), as previously observed by Wolf et al.

The number of VEGF-positive vessels in diabetic eyes was related to one of the biologic activities of VEGF, increased vascular permeability. Normally, retinal vessels are impermeable to macromolecules, but leakage of small molecules such as sodium fluorescein is a well-documented characteristic of early diabetic retinopathy. In our study, when all HSA-positive vessels were counted, regardless of the intensity of staining, we observed more HSA-positive vessels in diabetic than in nondiabetic eyes, but the difference was not statistically significant. However, HSA staining in nondiabetic eyes was mostly weak, as opposed to the intense staining pattern we saw in diabetic eyes, where,
FIGURE 8. Heparan sulfate proteoglycan (HSPG) immunoreactivity was observed in almost all blood vessel walls in diabetic eyes (A, case 5) as well as in nondiabetic eyes (B, case 20). In diabetic eyes, vascular endothelial growth factor (VEGF) immunoreactivity (C) appeared to be colocalized with HSPG, whereas in nondiabetic eyes (D) the patterns of VEGF immunostaining were not related to HSPG. Arrowheads indicate the same vessel in serial sections from each subject; magnification, ×115.

despite a lower total number of viable vessels (vWF positive), HSA immunoreactivity was greatly increased (see Figs. 2 and 3). We also observed colocalization of VEGF immunoreactivity with vessels prominently stained for HSA (see Figs. 2C and 2E) in diabetic eyes, suggesting that VEGF was associated with the increased permeability of albumin. These observations were supported by demonstration of a statistical correlation between the number of HSA-positive vessels and VEGF-positive vessels in diabetic eyes. In nondiabetic eyes, no such correlation was observed (see Fig. 5). This suggests that the increased permeability of diabetic retinal vessels is related to the presence of VEGF. Early research on VEGF has demonstrated that it induces a marked increase in vascular permeability (thus its original name, vascular permeability factor). The diffuse staining of the inner photoreceptor matrix in diabetic persons, and to a lesser degree in nondiabetic persons, may be a result of diffusion of blood plasma components from the choroid—in other words, a breakdown of the outer blood–retinal barrier.

Heparan sulfate proteoglycan staining, which was observed in almost all retinal vessels, is a result of the glycocalyx production by endothelial cells. Vascular endothelial growth factor is a heparin-binding protein and therefore binds to HSPG, which could serve as an extracellular storage depot. We have previously observed colocalization of bFGF, another heparin-binding growth factor, and HSPG in diabetic persons and demonstrated that digestion of HSPG results in loss of the growth factor. In the present study, there was a good statistical correlation between the distribution of HSPG-positive vessels and VEGF-positive vessels in diabetic eyes; in nondiabetic eyes, no correlation was observed. The pattern of HSPG immunoreactivity was similar in diabetic and nondiabetic eyes, but colocalization of HSPG immunoreactivity and VEGF immunoreactivity was observed only in diabetic eyes (see Fig. 8). This again suggests a role for endothelial cell HSPG as a storage depot for heparin-binding growth factors. Finally, the observation of comparable HSPG staining in diabetic and nondiabetic eyes but less VEGF immunoreactivity in nondiabetic eyes suggests that more VEGF was available overall in diabetic eyes. Our study does not address the source of the VEGF in retina, but Pe'er et al recently demonstrated increased VEGF messenger RNA in inner retinal neuronal elements in diabetic retinopathy.

The relation between VEGF expression and hyp-
Immunohistochemical Analysis of VEGF in Human Diabetic Retina

**DIABETICS:** $y = 2.308x - 8.232; r^2 = 0.760$

**NONDIADETS:** $y = -0.983x + 22.419; r^2 = 0.035$

Oxidative stress has been demonstrated with in vitro systems and in tumors in vivo. In our study, we could not establish a correlation between capillary dropout—presumed hypoxia and the numbers of VEGF-positive vessels in the areas of retina sectioned. This could be attributed to our limited sampling for ghost vessel analysis or to the sample selection for the study; the mean age of diabetic subjects was 69 years compared with 76 years in nondiabetic subjects, thus yielding a nondiabetic study group with subjects who perhaps had greater age-related loss of peripheral retinal vasculature. The fellow retinas from each subject were incubated for ADPase activity and demonstrated no angiopathic changes beyond severe nonproliferative retinopathy, suggesting another possible explanation for the low number of ghost vessels in our diabetic group. In six of the seven cases where no retinopathy was seen in the fellow retina incubated for ADPase activity, VEGF and HSA immunoreactivity was high in the cryopreserved eye (see Fig. 7). Four of these eyes were obtained from non-insulin-dependent diabetic persons and one from a relatively young (age 50 years) insulin-dependent diabetic person. Thus, the allegedly paradoxical correlation between high VEGF and HSA and no angiopathic changes in the fellow retina coincided with the medical history of the milder form of disease in four cases and short duration of the disease in one case. This suggests that increased VEGF immunoreactivity and the previously reported increased vascular permeability are early events in diabetic retinopathy. A recent study by Amin et al supports the idea that VEGF immunoreactivity is elevated in early stages of diabetic retinopathy. Furthermore, a study by Tolentino et al demonstrated that intravitreal injections of VEGF can induce two early changes that occur in diabetic retinopathy: capillary nonperfusion and increased vascular permeability.

This study has demonstrated that the number of vessels immunoreactive for VEGF is significantly elevated in diabetic eyes. Furthermore, the localization of VEGF immunoreactivity appears related to HSPG in the blood vessel wall; therefore, HSPG may serve as a storage depot for VEGF, as previously hypothesized for bFGF in diabetic retina. Elevated VEGF was associated with increased vascular permeability, a characteristic of early diabetic retinopathy. Our study did not define sites of VEGF production nor even the cause of increased VEGF production, but it does suggest that increased VEGF immunoreactivity and increased vascular permeability appear to occur early in diabetic retinopathy and that hypoxia, as indicated by capillary dropout, may not be the only determinant for increased VEGF production.

**Key Words**

Diabetic retinopathy, heparan sulfate proteoglycan, immunohistochemistry, vascular endothelial growth factor, vascular permeability

**Acknowledgments**

The authors thank Jingtai Cao and Ichiro Fukushima for their assistance in grading the angiopathy in the ADPase retinal preparations and their insightful discussions concerning the manuscript.

**References**

5. Plouet J, Moukadiri H. Characterization of the receptor to vasculotropin on bovine adrenal cortex-derived.


35. Senger DD, Ferruzzi CA, Fedor J, Dvorak HF. A highly conserved vascular permeability factor secreted by a...


