Upregulation of the Vascular Endothelial Growth Factor/Vascular Endothelial Growth Factor Receptor System in Experimental Background Diabetic Retinopathy of the Rat

Hans-Peter Hammes, Jihong Lin, Reinhard G. Bretzel, Michael Brownlee, and Georg Breier

Vascular endothelial growth factor (VEGF) is a major contributor to retinal neovascularization. The possible participation of VEGF and its high-affinity tyrosine kinase receptors, flk-1 and flt-1, in early background diabetic retinopathy was studied in the streptozotocin-induced diabetic rat model of experimental retinopathy using in situ hybridization, blotting techniques, and immunohistochemistry. Diabetic retinopathy was assessed by quantitative morphometry of retinal digest preparations. The number of acellular capillaries increased 2.7-fold in diabetic animals with diabetes' duration of 6 months compared with nondiabetic controls. VEGF expression was not detectable by in situ hybridization in nondiabetic rats but was highly increased in the ganglion cell layer and in the inner and outer nuclear layers of retinas from diabetic animals. VEGF protein was extractable only from diabetic retinas, and a strong immunolabeling was detected in vascular and perivascular structures. Increased flk-1 and flt-1 mRNA levels were also found in the ganglion cell and both nuclear layers of diabetic samples only. Dot blot and Western blot analyses confirmed the increase in flk-1 mRNA and protein in diabetic retinas. Also, flk-1 immunoreactivity was associated with vascular and nonvascular structures of the inner retinas from diabetic animals. These data obtained from a rodent model in which retinal neovascularization does not occur support the concept that the VEGF/VEGF receptor system is upregulated in early diabetic retinopathy. *Diabetes* 47:401–406, 1998

Diabetic retinopathy is the most common microvascular complication in patients with long-standing type 1 diabetes (1). Clinically, diabetic retinopathy is divided into two stages: background and proliferative retinopathy. Background diabetic retinopathy is characterized by increased vascular permeability and progressive vascular occlusion (2). Retinal ischemia due to capillary nonperfusion causes a compensatory formation of new blood vessels, which then enter the vitreous space. This second proliferative stage of diabetic retinopathy leads to visual impairment through bleeding or retinal detachment by accompanying fibrous tissues (3).

The level of several growth factors—such as basic fibroblast growth factor, insulin-like growth factor, and vascular endothelial growth factor (VEGF)—has been found to be elevated in eyes of patients with active proliferation, thereby suggesting a combination of angiogenic stimuli (4,5). Experiments in animal models of hypoxia-induced ocular neovascularization have shown that VEGF is upregulated severalfold before the formation of new blood vessels, and that blocking its action inhibits retinal neovascularization (6–9). The cellular response to VEGF is mediated by the high-affinity tyrosine kinase receptors VEGF receptor (VEGFR)1/flt-1 and VEGFR2/flk-1, whose expression is limited to the endothelium under physiological conditions (10–13). These data suggest that VEGF is the most important growth factor involved in the pathogenesis of the proliferative stage of retinopathy.

Several isoforms of the VEGF family are generated by alternative splicing from the same gene (14). In contrast to other endothelial growth factors, VEGF has a signal sequence, and its predominant isoform $VEGF_{165}$ is secreted by several cell types in the retina, including pericytes, ganglion cells, Müller cells, and the retinal pigment epithelium (6,15,16). VEGF is a specific mitogen for vascular endothelial cells and also a permeability factor (17–19). Its expression is induced by hypoxia, which regulates transcriptional activity and increased mRNA stability (20–22). Increased local production of VEGF has been associated with tumor neovascularization, developmental angiogenesis, and ischemia-induced proliferative ocular diseases such as diabetic retinopathy (4,23,24).

During early stages of background retinopathy, however, retinal digest preparations from humans and diabetic animal models have shown only scattered capillary occlusions. This minor degree of vascular occlusion may not be sufficient to consider hypoxia as a major stimulus for the observation of increased mitogenic activity at this very early stage of microangiopathy (25,26). In contrast, increased vascular permeability is a characteristic sign of early stages of diabetic retinopathy. Because VEGF is a vascular permeability factor, it may already be upregulated during early stages of diabetic retinopathy.

From the III Medical Department, Internal Medicine (H.-P.H., J.L., R.G.B.), Justus-Liebig University, Giessen, Germany; the Albert Einstein College of Medicine (M.B.), Bronx, New York; and the Max-Planck-Institute for Physiological and Clinical Research (G.B.), Bad Nauheim, Germany.

Address correspondence and reprint requests to Dr. Hans-Peter Hammes, III Medical Department, Justus-Liebig University, Rodthohl 6, 35392 Giessen, Germany. E-mail: hans-peter.hammes@innere.med.uni-giessen.de.

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ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

To gain further insight into the possible participation of the VEGF/VEGFR system in early background retinopathy (i.e., in the absence of retinal neovascularization), we studied in parallel the expression of VEGF and its receptors at the mRNA level and VEGF and flk-1 at the protein level in rats with a diabetes duration of 6 months.

RESEARCH DESIGN AND METHODS

Experiments performed in this study adhered to the Association for Research in Vision and Ophthalmology statement for the "Use of Animals in Ophthalmic and Vision Research."

Animals. Chronic hyperglycemia was induced in 6-week-old male Wistar (Charles River, Sulzfeld, Germany) rats by intravenous injection of 65 mg/kg body wt streptozotocin (STZ; Boehringer Mannheim, Mannheim, Germany). Diabetes was monitored consecutively by means of body weight and blood glucose determinations. In addition, GHb was determined at the end of the study using affinity chromatography (Glyc Affin; Isolab, Akron, OH). The study was terminated 6 months after STZ injection.

Retinal preparation. Eyes from nondiabetic ($n = 6$) and diabetic ($n = 5$) rats were obtained at the end of the study under deep anesthesia and embedded in OCT (Miles, Naperville, IL). Tissue sections (6 µm) were cut on a cryostat at –20°C and placed on poly-L-lysine–coated slides.

In addition, eyes were obtained after 12 weeks from two rats that had remained normoglycemic despite STZ injection (nonfasting blood glucose 6.02 ± 0.29 mmol/l [mean ± SD]).

Quantitation of acellular capillaries in retinal digest preparations. Contralateral eyes (normal, $n = 6$; diabetic, $n = 5$) were immediately fixed in 4% buffered formalin. Retinal digest preparation and quantitation of acellular capillaries were performed as previously described (27).

In situ hybridization. cDNA probes for VEGF flk-1 and flt-1 were prepared as previously described (13). Antisense and sense cRNA probes were generated by in vitro transcription using T3 or T7 RNA polymerases and labeled with biotin using the biotin RNA labeling kit (Boehringer Mannheim).

For in situ hybridization, 10 sections per group (from 6 normal and 5 diabetic eyes) were incubated at 55°C for 16 h with hybridization solution (50% formamide, 100 µg/ml salmon sperm DNA, 0.05 mol/l sodium phosphate, 0.6 mol/l NaCl, 5 mmol/l EDTA, 1 × Denhardt's solution, and 1 µmol/l dithiothreitol) containing 150 ng/ml of biotinylated VEGF c-RNA. Alternatively, sections were hybridized for 2 h at 55°C in a hybridization solution containing 500 ng/ml biotinylated flt-1 and flk-1-cRNA, respectively. The streptavidin-alkaline phosphatase system including 5′-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium chloride (Boehringer Mannheim) was used for detection and visualization. Negative controls included incubation with a sense ribonucleotide probe and omission of an antisense ribonucleotide probe.

Dot blot analysis of total retinal flk-1 expression. Retinas were isolated from freshly obtained eyes (two eyes from each group), homogenized in guanidinium thiocyanate buffer, diluted in TE buffer (containing 10 mmol/l Tris-HCl, pH 7.5, and 1 mmol/l EDTA), and centrifuged for 1 min at 13,000*g*). mRNA was extracted using the QuickPrep mRNA purification kit (Pharmacia Biotech, Munich, Germany). One microliter per dot (containing 5 ng mRNA) was transferred to a nitrocellulose membrane, heat fixed, prehybridized, and hybridized with the biotin-labeled flk-1 antisense probe described above. Equal loading was confirmed by measuring OD_{260nm} of retinal mRNA extracts, and lane loading differences were normalized using a β-actin cDNA probe (28). Hybridization signals were detected using the Nucleotide Labeling and Detection Kit (Boehringer Mannheim). Experiments were performed in triplicate. Blots were quantitated using image analysis–based densitometry (CUE-2; Olympus Opticals, Hamburg, Germany). Grey levels and areas of the blots were measured and normalized for mRNA loading as assessed by the actin signal.

Immunohistochemistry. Horizontal cryostat sections were used for VEGF immunohistochemistry (5–8 sections per animal). Briefly, 6-µm sections were cut through the outer part of the inner nuclear layer in parallel to the inner limiting membrane, so that a part of the outer capillary network could be visualized. Sections were incubated with a polyclonal rabbit anti-VEGF IgG raised against amino acids 1–20 of the NH_2-terminus of VEGF (5 µg/ml; Santa Cruz/IC Chemikalien, Ismaning, Germany). After exposure to a fluorescein isothiocyanate (FITC)–labeled secondary anti-rabbit antibody (1:30; DAKO, Hamburg, Germany), immunoreactivity was visualized by epifluorescence microscopy. For control, the primary antibody was replaced by a nonimmune rabbit IgG or omitted.

Vertical cryostat sections were used for flk-1 immunohistochemistry. Sections were incubated with a polyclonal antibody, raised against a peptide corresponding to amino acids 1326–1345 at the COOH-terminus of mouse flk-1 (5 µg/ml; WAK-Chemie, Munich, Germany) and reacted with an FITC-conjugated secondary anti-rabbit antibody (DAKO). Control experiments were performed as described above.

Retinal protein extraction. Retinal tissue was obtained from two normal and three diabetic rats. After isolation, each retina was powdered in liquid nitrogen and taken up in reducing SDS sample buffer containing 6 mol/l urea at a protein concentration of 100–200 mg/ml. After being boiled for 15 min and then ultrasonicated, samples were centrifuged at 10,000*g*, and supernatants were subjected to SDS-polyacrylamide-gel electrophoresis. After transfer of proteins to nitrocellulose, the filter was incubated with 0.5% nonfat dry milk and dissolved in phosphate-buffered saline (PBS) containing 0.1% Tween 20 for 30 min. Subsequently, the filter was incubated with the polyclonal anti-VEGF antibody (0.1 µg/ml), and the anti–flk-1 antibody (0.1 µg/ml) was dissolved in PBS containing 0.1% Tween and 0.5% NP 40 for 1 h at 22°C. VEGF and flk-1 were identified using the enhanced chemiluminescence Western blotting detection system (Amersham, Braunschweig, Germany). Experiments were performed in triplicate. Quantitative analysis of the flk-1 Western blot was performed essentially as described above, except that the results were normalized for protein concentrations of the retinal extracts (modified biuret method; Sigma, Deisenhofen, Germany).

Statistical analysis. Parameters are given as means ± SD. The significance of differences between groups was tested using one-way analysis of variance and the Student-Newman-Keuls test.

RESULTS

After 6 months of hyperglycemia, diabetic rats weighed 328 ± 51 g, their blood glucose levels were 24.4 ± 4.5 mmol/l, and their GHb was 14.4 ± 2.7%. All values were significantly different ($P < 0.001$) from the corresponding parameters of the nondiabetic control rats (body weight 531 ± 55 g, blood glucose 4.9 ± 0.4 mmol/l, GHb 4.5 ± 0.7%). The number of acellular capillaries was 17.1 ± 4.0 vs. 45.4 ± 10.2 per mm^2 of retinal area in nondiabetic and diabetic animals, respectively ($P < 0.001$).

VEGF expression. In situ hybridization for VEGF revealed a major increase of mRNA expression in different layers of the retinas of diabetic rats. VEGF expression was upregulated predominantly in the ganglion cell layer and in the inner and outer nuclear layers. A weak signal was also detectable in the retinal pigment epithelium (Fig. 1*B*). VEGF transcripts were not detectable in the retinas of nondiabetic rats (Fig. 1*A*).

VEGF immunoreactivity in nondiabetic rats was weak and present only in a minority of capillaries crossing the outer parts of the inner nuclear layer (Fig. 2*A*). No immunoreactivity was observed in the inner capillary network or the larger vessels (not shown).

By comparison, a strong VEGF immunoreactivity was seen in the vascular wall of capillaries from diabetic rats in the outer part of the inner nuclear layer (Fig. 2*B*) and in the closest proximity to the affected capillaries. This suggested that VEGF protein secreted by cells of the ganglion cell layer and the inner and outer nuclear layers accumulated in the vasculature of the inner retina. Although some immunolabeling was also present in the inner capillary network, no VEGF was detected in larger vessels (not shown).

Retinal protein extracts were performed to confirm the relative increase in VEGF protein levels in retinal tissue. It was found that, after a diabetes duration of 6 months, an increase in VEGF protein expression occurred, whereas no detectable VEGF was present in the retinal extracts in nondiabetic animals (Fig. 2*G*).

flk-1 expression. In parallel with the increase in VEGF transcription, flk-1 mRNA was upregulated in the retinas of diabetic rats (Fig. 1*E*). The majority of the signals were found in the ganglion cell layer and the inner and outer nuclear layers. No flk-1 mRNA was observed in any of the nondiabetic retinas tested (Fig. 1*D*). To confirm the increase of flk-1 expression, flk-1 mRNA dot blot was performed from retinal mRNA

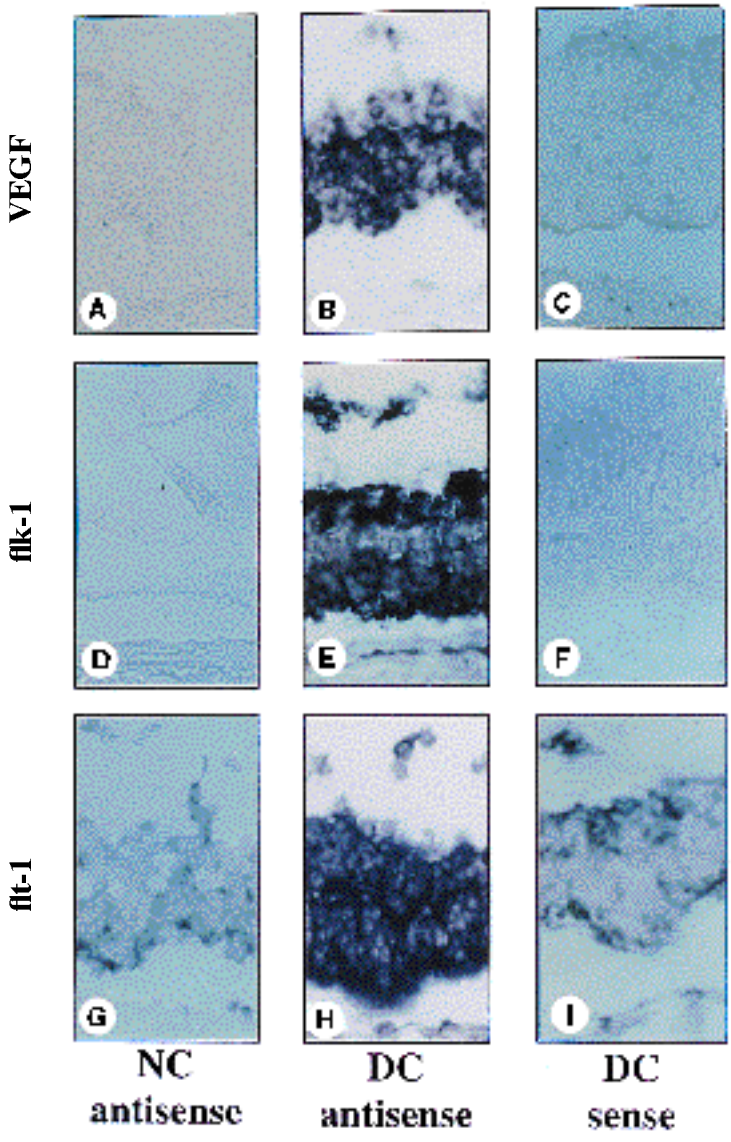

FIG. 1. Expression of VEGF, flk-1, and flt-1 mRNA in diabetic (DC) and age-matched nondiabetic (NC) rats. VEGF (*A*), flk-1 (*D*), and flt-1 (*G*) mRNA were not detectable in NC rats. VEGF mRNA expression was upregulated in the ganglion cell and inner and outer nuclear layers, and weakly in the retinal pigment epithelium of diabetic rats (*B*). In the same layers, strong signals were detected for flk-1 mRNA expression (*E*) and flt-1 expression (*H*) of diabetic rats. Sense control sections of diabetic rats are shown for VEGF (*C*), flk-1 (*F*), and flt-1 (*I*) (DC sense). Original magnification ×250.

extracts. Results shown in Fig. 2*H* indicate an almost 3.6-fold increase of signal in diabetic compared with nondiabetic retinal mRNA, as judged by densitometry.

Vertical cryostat sections were used to identify flk-1 immunoreactivity. In nondiabetic retinas, immunolabeling with the polyclonal antibody showed no discernible flk-1 protein expression (Fig. 2*D*). In diabetic retinas, apart from moderate immunofluorescence throughout the entire retina, an increased immunolabeling was found in structures traversing the inner plexiform layer, presumably capillaries, and in the ganglion cell and inner nuclear layers (Fig. 2*E*).

Retinal protein extracts from nondiabetic rats confirmed the immunohistochemical findings in that no apparent signal was detected by Western blot analysis (Fig. 2*I*). In contrast, protein extracts from diabetic rats showed a distinct band, confirming the upregulation of this receptor in the diabetic rat retina.

flt-1 transcription. In parallel with the increased expression of VEGF and flk-1 mRNA, a strong hybridization with flt-1 cRNA was detected in retinas of diabetic rats in the same layers as with flk-1. Again, the positive layers were the ganglion cell and inner and outer nuclear layers (Fig. 1*H*). In nondiabetic rats, only a weak reactivity was present in the outer nuclear layer (Fig. 1*G*). However, this reactivity was not different from the control hybridization using flt-1 sense probe (Fig. 1*I*), indicating that this reaction was nonspecific.

In addition, in situ hybridization for VEGF and flk-1 was performed in retinas from rats that received STZ but remained normoglycemic. Virtually no signal was observed in these sections, indicating that STZ did not affect VEGF and flk-1 expression (data not shown).

DISCUSSION

VEGF has been suggested to be the major factor in the initiation of advanced stages of diabetic retinopathy because it is induced by ischemia and its mitogenic effect is specific to endothelial cells.

Data from animal studies using a mouse model of hypoxia-induced retinal neovascularization support this concept, showing that VEGF expression in the ganglion cell layer and the inner nuclear layer precedes angiogenesis, and that therapies targeting the VEGF receptors and VEGF mRNA reduces the preretinal new vessel formation (7–9).

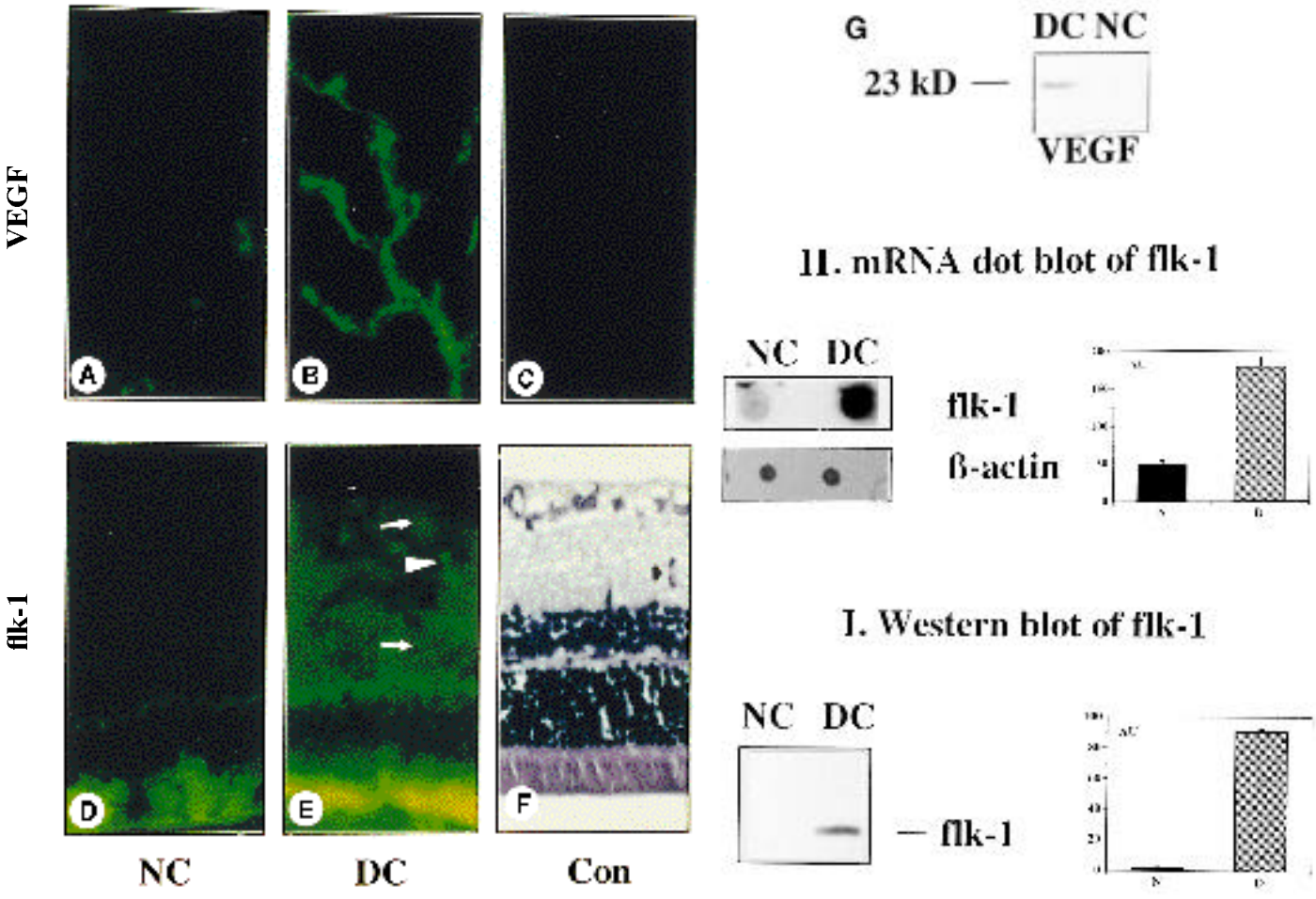

FIG. 2. Immunohistochemistry of VEGF (*A–C*) and flk-1 (*D* and *E*) of nondiabetic (NC) and diabetic (DC) rats. Vertical sections through the outer parts of the inner nuclear layer were used from NC (*A*) and DC (*B*) rats to locate VEGF protein. In diabetic retinas, a positive signal was found in capillaries and pericapillary areas (*B*). A representative control section of a diabetic rat omitting the primary antibody is given in (*C*). Immunohistochemistry of flk-1 in nondiabetic retinas was virtually negative (*D*). Immunolabeling for flk-1 protein was increased in structures traversing the inner plexiform layer, presumably capillaries (s), and in nonvascular structures of the ganglion cell layer and the inner nuclear layer (⇨). A paraffin section stained with hematoxylin eosin is shown in *F* to enable identification of structures traversing the inner plexiform layer as capillaries (◗). Original magnification ×400. Comparative dot blots of retinal mRNA extracted from NC and DC retina, blotted against flk-1 antisense probe, and the results of densitometry are given in *H*. Comparative immunoblots of retinal protein extracts blotted against a flk-1 antibody are shown in (*G*). Additional quantitative results of the densitometry are also given.

Results from this study show that VEGF expression is markedly elevated in several layers of the inner retina after even a relatively short diabetes duration. Even after 3 months of STZ-induced diabetes in the rat, a moderate increase of VEGF and flk-1 mRNA expression was observed in separate experiments (H.-P.H., J.L., R.G.B., M.B., G.B., unpublished observations).

Increased VEGF expression is accompanied by upregulation of the two VEGF receptors, flt-1 and flk-1, which appear to be present not only in the retinal vasculature in diabetes but also in the ganglion cell and inner and outer nuclear layers. Recent observations showing the expression of KDR and flt-1 in smooth muscle cells of the uterus and the expression of flk-1 in neural progenitor cells of the mouse retina are consistent with our data—that is, that the expression of these VEGF-receptors is not confined to endothelial cells (29,30). Because flk-1 protein was not observed in the retina and extracts from nondiabetic rats, it is assumed that its expression in the normal vasculature of the rat retina is below the detection limit of the assays used.

From our experimental data, it can be ruled out that alterations in the VEGF/VEGF receptor system were caused by the injection of STZ, since no increase in VEGF and flk-1 mRNA expression was observed in those rats that remained normoglycemic despite administration of high dosages of STZ.

The stimulus for the increase in VEGF/VEGFR expression before the establishment of hypoxia in diabetic retinopathy remains to be elucidated.

Retinas from rats with a diabetes duration of 6 months were not ischemic, as judged by the numbers of occluded capillaries. Although the extent of capillary dropout in diabetic rats was increased two- to threefold compared with nondiabetic controls, the extent of nonperfusion are less than in the hyperoxia-induced mouse model of retinal neovascularization, suggesting that mechanisms other than hypoxia stimulate retinal VEGF production. Although it cannot be excluded

with certainty from the experiments presented here that hypoxia is involved in the early induction of VEGF expression, a potential source of upregulated VEGF gene expression in diabetic tissue is the increased generation of reactive oxygen species (ROS) (31). ROS production in diabetes is the cumulative result of glucose autoxidation, autoxidation of glycated proteins, and advanced glycosylation end product binding to their cellular receptors. Experimental evidence for increased retinal ROS in diabetic rats has been presented (32), and the mechanism by which ROS increases VEGF gene expression has been determined (increased in VEGF mRNA stability) (31).

Recently, it has been reported that VEGF expression is upregulated in diabetic rats associated with increased vascular permeability (33). Although no data on the degree of hyperglycemia of the diabetic rats or the extent of capillary dropout were given in that study, VEGF was found predominantly in ganglion and Müller cells as well as to some extent in pericytes, smooth muscle cells, and astrocytes. It was concluded that extravasated plasma proteins may induce retinal neovascularization in conjunction with an increased VEGF-mediated mitogenic effect on endothelial cells. The diabetic rat model, however, reportedly never develops preretinal neovascularization (34). The reason for this is unclear at present. It has been speculated that diabetic rodents do not live long enough to experience advanced stages of retinopathy. It is also possible that intrinsic anti-angiogenic factors that counteract angiogenic stimuli are highly active in the rat eye, although direct evidence for such a hypothesis is lacking. The degree of stimulation through angiogenic factors and the maturity of the vessels involved may play a further determining role, since it is possible to induce retinal neovascularization in newborn but not in adult rats by cyclic hyperoxia/hypoxia exposure (35,36). Thus VEGF upregulation in the initial course of diabetes may occur for reasons other than in compensation for ischemia.

Upregulation of the VEGF receptor systems in retinas from chronic hyperglycemic rats is unprecedented. Surprisingly, flt-1 and flk-1 expression in diabetes is not restricted to the retinal vasculature. It appears that ganglion cells and presumably also Müller cells contain receptor mRNA, although translation into detectable protein was demonstrable only for flk-1, since an appropriate antibody for flt-1 is not yet available. As mentioned above, the ability of nonvascular cells to express VEGF receptors after stimulation in general has been described. Such stimulation can also result from malignant transformation (37–39). Mechanical or enzymatic stress involved in cell culture techniques is another nonspecific stimulatory factor for gene expression. In particular, cultured cells express VEGFRs, which they do not express under unstimulated in vivo conditions (40,41). Chronic hyperglycemia is likely to represent a stimulatory condition similar to those described above, as it has been shown that Müller cells transdifferentiate into a glial fibrillary acid protein–expressing phenotype and upregulate the low-affinity nerve growth factor receptor in diabetic rats (42). The upregulation of VEGF and its receptors in diabetic retinas may represent a similar example of stress-induced cell response.

The biological significance of the observed changes in the VEGF/VEGFR system is unclear. One possibility is that VEGF is a maintenance/survival factor for certain cell types and tissues that are damaged by the diabetic state.

These findings may have important implications for anti-VEGF strategies in diabetic retinopathy.

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