Rapid Publication

Loss of the Antiangiogenic Pigment Epithelium-Derived Factor in Patients With Angiogenic Eye Disease

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Retinal neovascularization characterizes proliferative diabetic retinopathy (PDR). Pigment epithelium-derived factor (PEDF) has been shown to be a major antiangiogenic growth factor in the mammalian eye. PEDF expression is suppressed by hypoxia, and changes in PEDF have been correlated to the development of retinal neovascularization in animal models of hypoxic eye disease. However, whether this concept of a reduced angiogenesis inhibitor holds true in humans is as yet unclear. In this study, we analyzed the in vivo regulation of PEDF in patients with and without hypoxic eye disease. We used immunoblots to measure PEDF in ocular fluids obtained from 64 nondiabetic and diabetic patients. In addition, immunohistochemistry of PEDF was carried out in specimens of normal human retinas and retinas with various degrees of diabetic retinopathy. The PEDF concentrations in patients with PDR ($P < 0.001$) or extensive nondiabetic retinal neovascularization caused by retinal-vein occlusion ($P < 0.001$) were lower than in control patients. Levels of PEDF were replenished in PDR patients with previous retinal scatter photocoagulation compared with PDR patients without previous photocoagulation ($P = 0.01$). Immunohistochemistry revealed an interstitial staining pattern as expected for a secreted protein, with an intense staining in retinas of patients without proliferative eye disease. However, in patients with PDR, little or no staining was detectable. Our data strongly support the concept that retinal angiogenesis is induced by loss of the major angiogenesis inhibitor in the eye, PEDF, in combination with an increased expression of angiogenic growth factors such as vascular endothelial growth factor. Our findings suggest that substitution of angiogenesis inhibitors may be an effective approach in the treatment of PDR. *Diabetes* 50:2641–2645, 2001

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EPC-1, early population doubling level cDNA-1; NPDR, nonproliferative diabetic retinopathy; NVD, new vessels on the disk; NVE, new vessels elsewhere; PDR, proliferative diabetic retinopathy; PEDF, pigment epithelium-derived factor; PRP, previous retinal photocoagulation; VEGF, vascular endothelial growth factor.

The control of retinal angiogenesis is of critical importance for the preservation of vision. Retinal neovascularization characterizes proliferative diabetic retinopathy (PDR), which is still one of the most common causes of blindness worldwide. Retinal ischemia induces intraocular neovascularization, presumably by stimulating the expression of angiogenic growth factors and by inhibiting the release of antiangiogenic cytokines (1,2). Vitreal levels of angiogenic growth factors have been shown to be directly associated with the degree of retinal angiogenesis (3,4). The ability to monitor and grade retinal angiogenesis within the eye as well as the ability to aspirate vitreous, which is known to contain retina-derived growth factors in direct association to the stage of retinal angiogenesis, makes the eye an ideal setting in which to investigate the delicate balance of new vessel growth and the influence of specific growth factors in vivo in humans.

Pigment epithelium-derived factor (PEDF) protects cerebellar granule cells against neurotoxic agents (5) and is also called early population doubling level cDNA-1 (EPC-1), reflecting its upregulation during cell cycle arrest (G_0) in young but not in senescent cultured fibroblasts (6). Recently, PEDF has been shown to be a highly effective inhibitor of angiogenesis in animal and cell culture models. The production of PEDF was decreased by hypoxia (7), which is also a central pathogenic stimulus in PDR. Immunoneutralization of PEDF diminished the ability of cadaveric human vitreous to inhibit migration of endothelial cells, thereby demonstrating that a loss of PEDF is functionally important in mediating angiogenic properties of human vitreous ex vivo. Most importantly, systemically administered PEDF prevented aberrant blood vessel growth in a murine model of ischemia-induced retinopathy (8). However, no information is yet available about the presence and regulation of PEDF in vivo in humans, particularly in hypoxia-induced proliferative retinopathy. If PEDF is involved in the control of retinal angiogenesis in humans, one would expect that PEDF is decreased in the ocular fluids of patients with hypoxia-induced proliferative retinopathy and that PEDF levels increase after at least

partially successful therapy, such as retinal photocoagulation. In this study, we attempted to ascertain whether intraocular concentrations of PEDF correlated with the gree of retinal neovascularization by measuring PEDF in the ocular fluid of 64 patients. We also investigated whether retinal scatter photocoagulation is capable of replenishing PEDF in the ocular fluid of patients with PDR. Spatial and temporal changes in the expression of retinal PEDF were determined by immunohistochemical localization of PEDF in the human retinas of patients with different degrees of diabetic retinopathy.

RESEARCH DESIGN AND METHODS

Vitreous was obtained from 64 patients (32 women and 32 men). Patients without proliferative retinal disease (control subjects: $n = 19$, 6 women and 13 men, mean age 70 ± 3 years) were compared with patients with PDR ($n = 37$, 17 women and 20 men, mean age 61 ± 2 years, 6 patients with type 1 diabetes, 31 with type 2 diabetes, HbA_{1c} $7.8 \pm 0.1\%$) and patients with extensive nondiabetic neovascularizing eye disease caused by central-vein occlusion (Rubeosis; $n = 8$, 2 women and 6 men, mean age 71 ± 3 years, no diabetes). A total of 27 patients with PDR had retinal photocoagulation before vitrectomy (PDR + previous retinal photocoagulation [PRP]), whereas 10 patients with PDR had no previous photocoagulation (PDR − PRP). PDR was considered to be active if there was extensive retinal neovascularization represented by perfused, multibranching preretinal capillaries and to be quiescent if mainly nonperfused or gliotic vessels were present. Altogether, 15 patients with PDR had active neovascularization, whereas 22 patients had quiescent retinal angiogenesis. A total of 13 patients with PDR + PRP had new vessels elsewhere (NVE), 3 had new vessels on the disk (NVD), and 11 had NVE + NVD. Five patients with PDR − PRP had NVE, four had NVD, and one had NVE and NVD. Age, HbA_{1c}, and duration of diabetes did not differ significantly between patients with PDR + PRP and PDR − PRP (age 61 ± 4 and 61 ± 2 years, HbA_{1c} 7.6 ± 0.3 and $8.2 \pm 0.4\%$, duration of diabetes 18.3 ± 2 and 17 ± 4 years, respectively). Undiluted samples of human vitreous were obtained during pars plana vitrectomy. Samples were aspirated under standardized conditions directly above the retina at the beginning of surgery and prepared as previously described (2). Ocular neovascular activity was determined by fluorescein photography, via slit lamp examination, or by the surgeon at the time of surgery.

Specimens for immunohistochemistry were obtained from the National Disease Research Interchange (NDRI), Philadelphia, Pennsylvania. Eyes were enucleated and fixed in 10% neutral buffered formalin within 10 h post mortem. Examination of the posterior segment was performed by an experienced ophthalmologist using a Zeiss Stemi SV8 zoom dissecting microscope. Eyes were categorized as follows ($n = 5$ for each group): normal (*A*); diabetic without ocular abnormalities (*B*); diabetic with intraretinal changes but no evidence of PDR (*C*); diabetic with PDR (*D*); and diabetic with scatter laser photocoagulation and no evidence of residual PDR (*E*). Samples were prepared, and criteria for categorization were chosen as previously described (9).

Classification of specimen was performed before the experimental part of the study. The study was approved by the Ethical Committee of the University of Bochum, and informed consent was obtained from all patients included.
Western blot. PEDF was quantified by Western blotting using polyclonal PEDF-specific antibodies (anti-PEDF), which were raised as previously described (10). Blots were analyzed automatically by a digital imaging system with standardized imaging values, thereby obtaining observer-independent quantification of the band intensities. The samples were compared with defined quantities of purified human PEDF, which was run as an internal standard on every gel. The internal standard was engineered by transfecting a human PEDF cDNA (with a 6xHis tag cloned into CEP4) (Invitrogen) into human embryonic kidney cells as previously described (7). Recombinant PEDF was enriched from the conditioned media with the QIAexpress system (Qiagen, Hildesheim, Germany) and quantified using the Bradford assay (11).
Immunohistochemistry. Primary antibody (anti-PEDF, 1:300 dilution) was incubated for 60 min. Detection was performed with an alkaline phosphatase–based system (LASB+; Dako, Glostrup, Denmark). Staining procedures were performed under standardized conditions, and sections were counterstained with Mayer's hematoxylin. Negative controls were incubated without primary antibody or with primary antibody after preabsorbtion with recombinant PEDF. The intensity of staining was graded qualitatively as background (*0*), weak (*1*), moderate (*2*), or intense (*3*) by a blinded investigator without
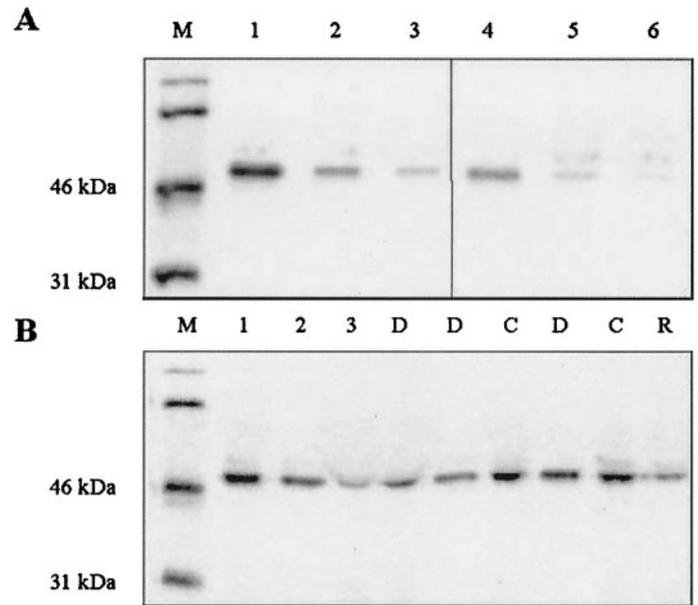
FIG. 1. Western blots with a polyclonal PEDF/EPC-1 antibody. *Lanes 1-3* represent a typical standard curve with a dilution of recombinant PEDF (*lane 1*, undiluted; *lane 2*, 1:2 dilution; *lane 3*, 1:4 dilution). M, molecular size marker. *A*: The PEDF bands in *lanes 1--3* were reduced or disappeared after preincubation of the antibody with recombinant PEDF (*lanes 4--6*), thereby demonstrating the specificity of the reaction. *B*: Western blot of vitreal samples of control subject (*C*), patients with PDR (*D*), and patients with severe intraocular neovascularization (Rubeosis iridis) caused by central-vein occlusion (*R*).

knowledge of the clinical data. An average score of staining was calculated within each group.
Statistical analysis. Data are reported as the mean ± SE. The Mann Whitney *U* test was used to compare quantitative data with unequal distributions. The correlation between variables was calculated by linear regression analysis of untransformed values. A level of $P < 0.05$ was considered significant.

RESULTS

Vitreal levels of PEDF were determined by immunoblot (Fig. 1). We detected a protein band of 50 kDa corresponding to the predicted molecular mass of PEDF. The band disappeared or was diminished after preincubation of the antibody with a previously enriched recombinant PEDF, thereby demonstrating specificity of the reaction. Recombinant PEDF occurred as a single band on a SDS-polyacrylamide gel as investigated by Ponceau S staining after immobilization on a nitrocellulose filter.
Vitreal PEDF levels are decreased in PDR. The intraocular levels of PEDF were determined by Western blot analysis and then quantified based on an internal standard of purified human recombinant PEDF (Fig. 2). The results suggest that the PEDF levels were significantly decreased in patients with PDR (20 ± 0.5 nmol/l, $n = 37$; $P < 0.001$) and patients with central-vein occlusion resulting in extensive neovascularization (17.6 ± 0.3 nmol/l, $n = 8$; $P < 0.0001$) compared with control subjects (23.7 ± 0.7 nmol/l, $n = 19$). Furthermore, patients with quiescent PDR had unchanged PEDF levels (22 ± 0.6 nmol/l, $n = 22$; $P = 0.06$) compared with control subjects, whereas patients with active PDR (17.2 ± 0.5 nmol/l, $n = 15$) had PEDF levels comparable with those of patients with Rubeosis. PEDF levels of patients with active PDR were significantly lower than those of control subjects ($P < 0.0001$) and patients with quiescent PDR ($P < 0.0001$).
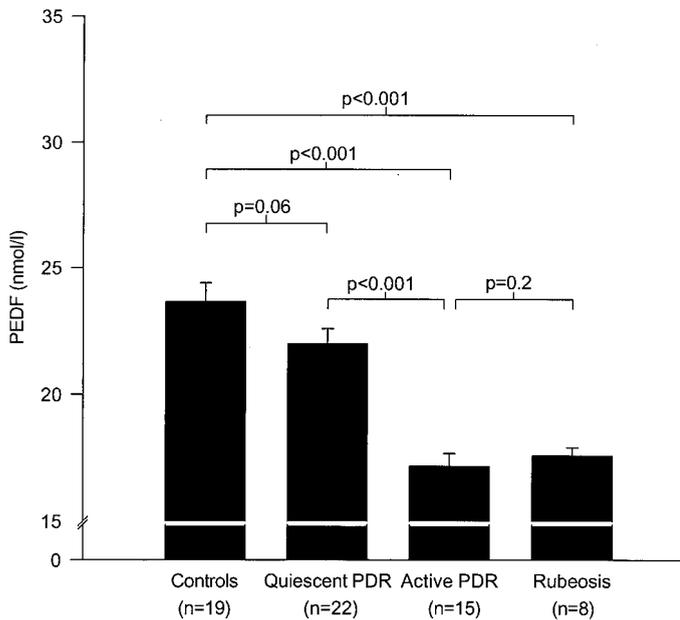
FIG. 2. Levels of vitreal PEDF in patients with proliferating eye disease. PEDF levels in intraocular samples from numerous patients were determined by Western blot analysis and compared with a standard concentration of purified human recombinant PEDF. The influence of intraocular activity was investigated by comparing levels of intraocular PEDF in patients with different degrees of neovascular activity: control subjects without angiogenesis, patients with quiescent PDR, patients with active PDR, and nondiabetic patients with extensive retinal neovascularization caused by central-vein occlusion (Rubeosis).

Photocoagulation replenishes intraocular levels of PEDF. Previous photocoagulation was associated with reduced neovascular activity (Fig. 3). Although 70% of the patients without prior photocoagulation (PDR − PRP) suffered from active angiogenesis, only 30% of the patients with PDR + PRP had active neovascularization. Patients with PDR + PRP had higher concentrations of PEDF ($n = 27$, 20.9 ± 0.7 nmol/l; $P = 0.01$) compared with patients with PDR − PRP ($n = 10$, 17.7 ± 0.3 nmol/l). However, PDR concentrations of patients with previous photocoagulation were still clearly below levels of control patients ($P = 0.007$).

PEDF levels are associated with the localization of retinal neovascularization. Taking all patients into account, levels of PEDF correlated significantly with the localization of retinal neovascularization. Patients with NVE and NVD (18 ± 0.4 nmol/l, $n = 20$) had decreased levels compared with control patients without proliferation (23.7 ± 0.7 nmol/l, $n = 19$; $P < 0.001$) and patients with NVE only (21 ± 1 nmol/l, $n = 18$; $P = 0.02$) (Fig. 4). Patients with NVE or NVD only (19 ± 1 nmol/l, $n = 7$) had lower levels than control patients ($P = 0.053$ and $P = 0.002$, respectively). We found no correlation between vitreal levels of PEDF and sex, duration of diabetes, HbA_{1c}, or age of the patients.

PEDF-specific immunohistochemistry of human retinas with different stages of diabetic retinopathy. To obtain data about spatial and temporal changes of PEDF expression in the course of diabetic retinopathy, 25 specimens of human retina were examined by immunohistochemistry. Our results revealed an interstitial accumulation of PEDF in the eyes of control subjects (Fig. 5A), patients

with diabetes without ocular abnormalities (Fig. 5B), and patients with nonproliferative diabetic retinopathy (NPDR) (Fig. 5C), thereby confirming the murine staining pattern previously described (7). We qualitatively assessed the staining for each section (as described in RESEARCH DESIGN AND METHODS), and our results show that intraretinal intensity of staining was nearly abolished in patients with PDR (mean 0.4 [range 0–1]) (Fig. 5D) compared with control subjects (2.2 [1–3]), patients with diabetes without ocular disease (1.6 [1–2]), and patients with NPDR (1.2 [1–2]), despite unchanged intensity of unspecific staining of the fibrous tissue. Patients with previous scatter photocoagulation resulting in quiescent PDR had weak intraretinal immunochemical staining that was, on average, slightly more intense (1.0 [0–2]) (Fig. 5E) than that of patients with active PDR.

Taken together, our results demonstrate a significant intraocular loss of the angiogenesis inhibitor PEDF in patients with neovascularizing eye disease such as PDR. Intraocular levels of PEDF strongly correlate with the degree of retinal neovascularization. In addition, we demonstrate that retinal scatter photocoagulation, the treatment of choice for patients with diabetic retinopathy, replenishes concentrations of PEDF in the eye. Changes of vitreal levels are confirmed by immunohistochemistry, which reveals an interstitial staining pattern as expected for a secreted protein.

DISCUSSION

The switch to an angiogenic phenotype of proliferating tissues requires both upregulation of angiogenic stimulators and downregulation of angiogenesis inhibitors. An elevated expression of angiogenic growth factors such as vascular endothelial growth factor (VEGF) in patients with retinal neovascularization has been previously demon-
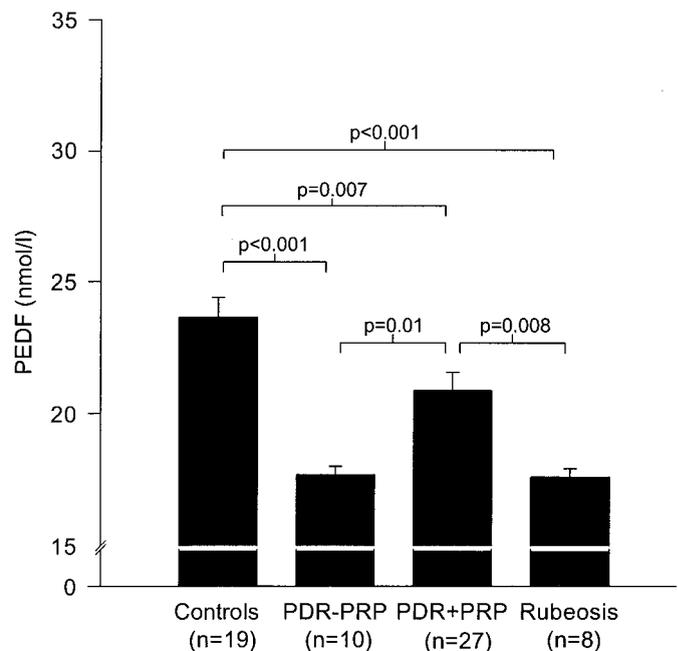
FIG. 3. Levels of vitreal PEDF depend on previous photocoagulation. PEDF levels were determined as described in Fig. 2 for patients with PDR − PRP, PDR + PRP, and Rubeosis, as well as nondiabetic patients with extensive retinal neovascularization due to central vein occlusion (control subjects).
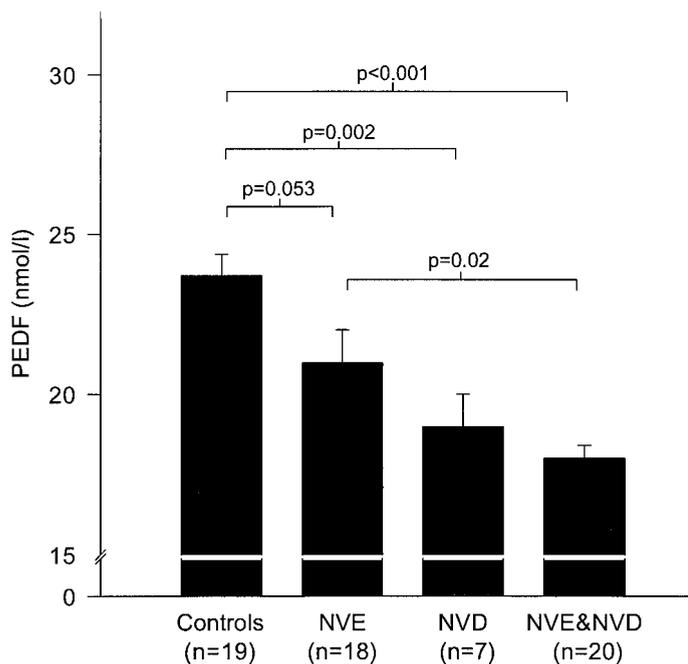
FIG. 4. Concentrations of PEDF in human vitreous depending on localization of neovasularization. PEDF levels were determined as described in Fig. 2. Levels of PEDF correlate with the localization of retinal neovascularization. We compared patients without retinal angiogenesis (control subjects), patients with NVD, those with NVE, and those with both NVE and NVD.

strated (4). Additionally, decreased expression of VEGF was observed in patients with reduced neovascular activity after panretinal photocoagulation (2). The question targeted by this study was whether a loss of angiostatic growth factors such as PEDF is critical in the development of retinal neovascularization in vivo in humans. We found PEDF concentrations in ocular fluid to be lower in patients with active neovascularization than in control subjects without retinal angiogenesis. The vitreal data are confirmed by the results of immunohistochemistry showing almost no staining in patients with active proliferation compared with a strong intraretinal staining in control patients. These results demonstrate regulation of the major intraocular angiogenesis inhibitor PEDF in vivo depending on the stage of retinal ischemia. The data support the concept that induction of angiogenesis in the human eye requires not only elevation of angiogenic growth factors such as VEGF (4) but also a decrease in angiogenesis inhibitors such as PEDF (1,7). PEDF has been proposed to be an age-dependent regulated protein (10). However, our data do not support this concept, although the number of control patients in our study may be too small to definitively answer this question.

We found that intraocular PEDF levels were reduced in nondiabetic patients with severe retinal ischemia caused by central-vein occlusion. Therefore, hypoxia rather than hyperglycemia promotes intraocular reduction of PEDF in humans. Our immunohistochemical findings show a small reduction in staining intensity in diabetic patients without retinal alterations and in diabetic patients with nonproliferative abnormalities (such as microaneurysms) compared with control subjects. These results suggest that glycemic control might also influence the expression of PEDF in the eye. Because of technical reasons in regard to

quantification of immunohistochemistry in general, we cannot fully exclude small differences in the expression of PEDF in NPDR compared with control patients. Even such small differences might be relevant in the early stages of diabetic retinopathy, as suggested by data showing PEDF-dependent functional changes of retinal vessels (L.P. Aiello, Boston, MA; personal communication).

An important observation of this study was that patients with quiescent retinal neovascularization who mostly had retinal photocoagulation before intraretinal surgery had higher levels of PEDF compared with patients with active neovascularization without previous photocoagulation. Retinal photocoagulation induces regression of retinal neovascularization and has been shown to be associated with a reduction in the incidence of severe visual loss and retinal neovascularization (12). In our study group, patients with previous photocoagulation had reduced neovascular activity compared with patients without prior photocoagulation, suggesting that the positive effects of retinal photocoagulation are mediated at least in part by the reestablishment of near-normal PEDF levels. Presumably, a reduction in retinal ischemia after photocoagula-
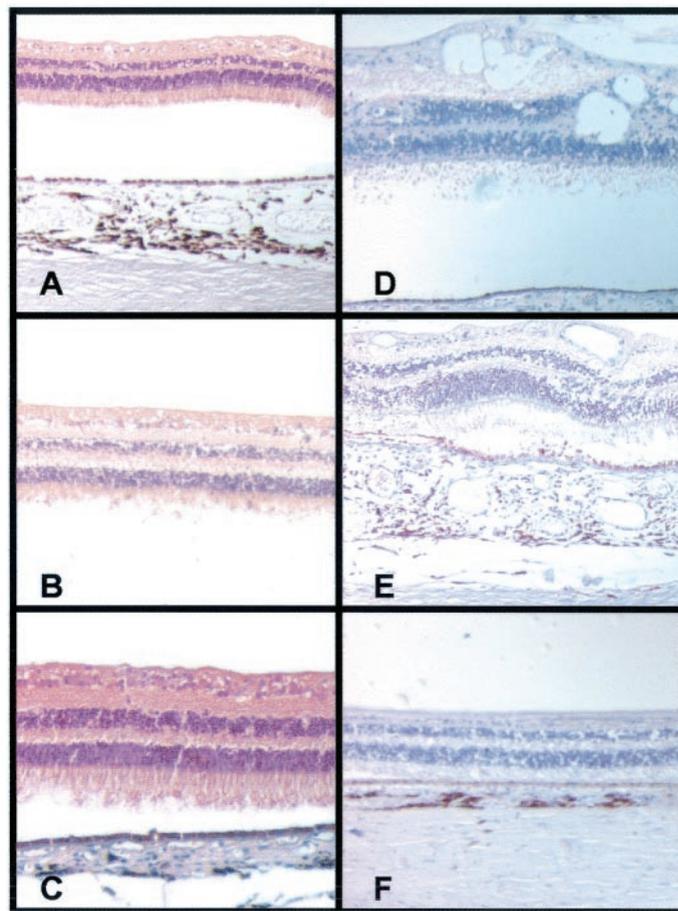
FIG. 5. PEDF protein expression in retinal samples from patients with different stages of diabetic retinopathy. Human retinas were examined by immunohistochemistry for PEDF expression. Staining patterns for representative sections are shown for control subjects (A), patients with diabetes without ocular abnormalities (B), and patients with NPDR (C) compared with patients with PDR (D) and patients with quiescent PDR after retinal photocoagulation (E). Specificity of reaction was demonstrated by the absence of staining after incubating sections with the primary antibody that was preabsorbed with the recombinant antigen (F).

tion increases expression of angiogenesis inhibitors such as PEDF, thereby further suppressing neovascular activity. Indeed, PEDF expression was initially induced by hyperoxia in neonatal mice (7). However, the patients in our study still exhibited intraocular proliferative activity requiring intraocular surgery. PEDF concentrations of patients after retinal scatter photocoagulation remained below those of control patients, thereby possibly explaining further existing proliferative activity in the subjects investigated.

A receptor for PEDF has not yet been identified, although radio-ligand binding studies in retinoblastoma cells and cerebellar granule neurons suggest a PEDF-specific receptor (13). Until now there has been no information about binding properties of putative receptors on vascular cells, putative binding proteins, or specific biological activities on different vascular cell types. Intraocular levels in mice are as high as 90 nmol/l. Despite these comparably high levels, systemically administered PEDF was able to completely inhibit aberrant retinal angiogenesis in a model of ischemia-induced proliferative retinopathy (8). This clearly indicates that increasing PEDF levels in the murine eye by systemic substitution is therapeutically effective. Our results with a loss of PEDF in humans strongly suggest that a similar PEDF-based treatment might be a promising therapeutic approach in patients with neovascularizing eye disease. Clearly, further investigations are needed to identify the exact mechanisms of PEDF release, PEDF-induced biological effects, and possible PEDF binding to putative binding proteins in the vitreous, such as that described for IGFs.

In conclusion, PEDF meets the criteria hypothesized for an ischemia-suppressed antiangiogenic factor (1). This principle, with obvious therapeutic impact, has been confirmed in animal studies (8). Here we suggest that the loss of a major angiogenesis inhibitor in the eye, PEDF, has a central role in vivo in humans in mediating the angiogenic response of retinal ischemia, such as that seen in PDR and other ischemic retinal disorders. In addition to the previously observed changes in angiogenic growth factors such as VEGF, our data support the hypothesis that an imbalance in the angiogenic ratio between angiogenic and antiangiogenic growth factors contributes significantly to the development of retinal neovascularization. Our data might potentially induce further investigations into the effectiveness of PEDF substitution in humans. Further characterization of ischemia-regulated PEDF expression and its biological effects should offer hopeful new therapeutic approaches to prevent blindness in patients with neovascularizing eye disease.

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