Regulation of Pigment Epithelium–Derived Factor Production and Release by Retinal Glial (Müller) Cells under Hypoxia

Johannes Lange, Yousef Yafai, Andreas Reichenbach, Peter Wiedemann, and Wolfram Eichler

Purpose. To assess the regulation of pigment epithelium–derived factor (PEDF) production by retinal Müller glial cells, especially under ischemic or hypoxic conditions.

Methods. PEDF was determined in surgically excised retinal tissue originating from patients with ischemic diabetic retinopathy and in primary guinea pig Müller cell cultures exposed to the protein synthesis inhibitor cycloheximide (CHX) and to hypoxia. PEDF production and secretion were studied by immunohistochemistry, quantitative RT-PCR, ELISA, fluorescence-activated cell sorter analysis, and Western blotting.

Results. Gliotic Müller cells displayed decreased PEDF immunoreactivity in fibrovascular tissue from patients with diabetes compared with tissue from subjects with pathologic myopia. In Müller cell cultures, CHX treatment resulted in an increase, whereas mild hypoxia (2.5%–10% O_2) induced a decrease, of PEDF mRNA and protein levels. However, strong hypoxia (0.2% O_2) induced an upregulation of PEDF mRNA expression and resulted in only slightly reduced PEDF levels after 24 hours, detected as either a released, soluble, or cell surface-linked protein.

Conclusions. These results suggest that under certain conditions including mild hypoxia, Müller cells synthesize a protein factor that downregulates PEDF expression or its turnover. Generally, the cells appear to generate a biphasic response to hypoxia. In moderate hypoxia, PEDF is downregulated such that the VEGF-to-PEDF ratio increases (and angiogenesis is facilitated). During severe (or chronic) oxygen deficiency, however, the PEDF decline is arrested or even reversed; thus, the neurotrophic effects of PEDF remain available. (*Invest Ophthalmol Vis Sci.* 2008;49:5161–5167) DOI:10.1167/iovs.08-2201

Pigment epithelium–derived factor (PEDF) is a secreted 50-kDa glycoprotein that was first identified in conditioned media of cultured fetal human retinal pigment epithelial (RPE) cells.[1] Although PEDF apparently is widely expressed, various studies have elucidated that this factor is particularly important for the tissue integrity of the neural retina. On the one hand, PEDF has neurotrophic and neuroprotective functions. This was demonstrated by the use of various animal models of ocular diseases in which PEDF protected retinal neurons from light-induced damage,[2] from oxidative cell death induced by hydrogen peroxide[3] in ischemic retinal injury,[4,5] and from apoptotic photoreceptor loss in genetic models of photoreceptor degeneration.[6] On the other hand, PEDF has been characterized as a highly potent inhibitor of angiogenesis in the eye compared with other endogenous antiangiogenic molecules.[7] Several studies indicated that PEDF is able to block endothelial cell migration[7] and proliferation[8–10] in vitro, and PEDF was also found to effectively inhibit neovascularization in animal models of ischemic retinopathies[11] and in patients with neovascular age-dependent macular degeneration.[12]

Vascular insufficiency and the development of retinal tissue ischemia or hypoxia are important pathogenic determinants in ocular disorders and diseases. These conditions are associated with the initiation of pathologic retinal neovascularization[13,14] but may also be accompanied by apoptotic neuronal cell death.[15,16] Both are important threats to vision. Indeed, ischemic proliferative retinopathies such as proliferative diabetic retinopathy (PDR) are the leading causes of blindness among the working-age population in modern societies.[17] Retinal neovascularization involves the activity of angiogenic and antiangiogenic mediators, their cell-associated receptors, various cytokines, and extracellular proteases (for a review, see Lee et al.[17]). Hypoxia and ischemia stimulate an upregulated expression and release of vascular endothelial growth factor-A (VEGF-A), a key proangiogenic molecule in retinal neovascularization.[13,14,18–20] VEGF-A exerts antiapoptotic, chemotactic, mitogenic, and proinflammatory activities,[21–24] and emerging evidence suggests that it also displays marked neurotrophic and neuroprotective properties.[25] Concomitantly, PEDF release from retinal cells becomes downregulated, providing a permissive condition for retinal neovascularization.[26] Thus, there seems to be a counterregulation of PEDF and VEGF-A release from ocular cells in neovascular retinal diseases such as PDR[27–31] such that intraretinal VEGF-A levels increase along with concomitantly decreased PEDF concentrations.[26]

Müller glial cells have important functions in the retina, regulating vasculogenesis, angiogenesis,[19,32] and tissue homeostasis.[33] One important facet of Müller cells is related to their ability to modulate retinal VEGF-A and PEDF levels. Indeed, the expression of VEGF-A mRNA and protein in Müller cells is stimulated after exposure to hypoxia,[19,32,34] suggesting that these glial cells are essential players in adjusting the retinal balance between proangiogenic and antiangiogenic mediators. In addition to their angioregulatory function, Müller cells are likely to exert important activities in controlling the survival of retinal neurons because Müller-cell derived neurotrophic and neuroprotective mediators (such as PEDF) can substantially support neuronal survival. It is this consideration that permits our hypothesis that in cases of hypoxia or ischemia, PEDF regulation by Müller cells has to comply with apparently conflicting requirements. PEDF levels should be low enough to

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tolerate the stimulation of angiogenesis to improve supply with nutrients but high enough to allow the survival of neurons. Because almost nothing is known about the regulation of PEDF synthesis and the release by Müller cells, we asked whether PEDF levels decreased in response to hypoxia in dedifferentiated Müller cell–derived cells within proliferative fibrovascular tissue in diabetic retinopathy and in primary Müller cell cultures from healthy adult guinea pigs. This study was aimed at an improved understanding of those conditions that ensure appropriate intraretinal PEDF levels, perhaps offering a therapeutic clue for PEDF-aided therapy of ocular neovascular disorders.[11,12,35]

Materials and Methods

Surgically Obtained Fibrovascular Tissue from Human Eyes

The use of human tissue conformed to the Declaration of Helsinki for biomedical research involving human subjects and was approved by the local University of Leipzig Human Ethics Committee. Informed written consent was obtained from the patients. To determine PEDF expression in Müller cell–derived dedifferentiated cells in proliferative retinopathies, we investigated surgically excised tissue from human patients. Two types of tissue were studied, retinal neovascular membranes originating from patients with proliferative diabetic retinopathy (PDR), considered a hypoxic tissue,[36] and fibrovascular retinal tissue from patients without diabetes but with subfoveal choroidal neovascularization caused by pathologic myopia. These samples were taken as controls with respect to hypoxia because hypoxia is not thought to be a main factor in this disease.[37,38] All other experiments were performed on primary Müller cell cultures.

Cell Cultures and Reagents

Retinal glial (Müller) cells were isolated from guinea pig retinas, as reported previously,[39] and were cultured for 7 days before the experiments. Identity of the cells was verified immunocytochemically with the use of polyclonal antibodies against vimentin (Biomeda, Foster City, CA) and glial fibrillary acidic protein (Dako, Hamburg, Germany). Müller cells were routinely cultured at 37°C, 5% CO_2, 95% air in Dulbecco modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 U/mL penicillin/100 mg/mL streptomycin, and they were allowed to grow to approximately 90% confluence. Before the experiments, the Müller cells were starved in serum-free DMEM for at least 4 hours. Cells were incubated for 24 hours with serum-free DMEM, either under normoxia (95% air, 5% CO_2) or hypoxia (0.2%–10% O_2, 5% CO_2) or in the presence of the protein synthesis inhibitor cycloheximide (CHX; 10 μg/mL). No indication of cellular damage was found in the cultures after hypoxia or in the presence of the inhibitor tested, as assessed by a dye exclusion assay using trypan blue, which is incorporated by nonviable cells but excluded from living cells (data not shown).

Immunohistochemistry

Neovascular complexes of patients with PDR and nonischemic high myopia (hereafter referred to as fibrovascular membranes) were obtained from patients undergoing retinal surgery. Surgical specimens were fixed in ice-cold acetone immediately after excision. Thawed tissue sections were rehydrated with PBS and treated with PBS/1% dimethyl sulfoxide/0.3% Triton X-100/5% normal goat serum (staining buffer). Samples were then incubated overnight (4°C) with polyclonal rabbit anti–PEDF (diluted 1:500; BioProducts, Middletown, MD) antibody. This antibody was previously demonstrated to bind PEDF from different sources, as judged by Western blot analysis,[10] and it abrogated the inhibitory effects of PEDF on VEGF-A–induced phosphorylation of ERK-1 and ERK-2 in bovine retinal endothelial cells and on endothelial cell proliferation.[40] Monoclonal mouse antivimentin (diluted 1:400; Immunotech, Beckman Coulter, Krefeld, Germany), anti–human cellular retinaldehyde-binding protein (CRALBP; clone B2, 1 μg/mL; a kind gift of John C. Saari, Department of Ophthalmology, University of Washington, Seattle, WA)[41] or anti–glial fibrillary acidic protein (GFAP, diluted 1:500; clone 6F2; Dako)[42] antibodies were directed against known markers of glial Müller cells. Monoclonal antibodies were added together with anti–PEDF antibody to identify glial cells in double-staining experiments. Specimens were washed with PBS/1% BSA and then incubated with cyanogen (Cy) 2-conjugated goat polyclonal anti–rabbit IgG and Cy 3-conjugated anti–mouse IgG (Dianova, Hamburg, Germany; both diluted in staining buffer). Then samples were counterstained (Hoechst 33342, 5 μg/mL; Sigma), covered by a nonfluorescent sealant and were examined under a fluorescent microscope (Axioskop; Carl Zeiss, Oberkochen, Germany) equipped with a digital camera. A scoring system implicating staining intensity was used for determining PEDF expression in membrane derived from patients with diabetes and patients with myopia, respectively. Four categories of staining were defined: no (0), faint (1), modest (2), and intense (3) stain. The intensity of staining in each of these categories was assessed by the authors (YY, JL, WE) without knowledge of the clinical or pathologic data for the particular sample.

Cell Surface Immunofluorescence Staining

Association of PEDF with the surface of Müller cells was determined by flow cytometry. Guinea pig Müller cells were detached with HBSS/1 mM EDTA, washed, and resuspended in HBSS/1% BSA supplemented with 10 mM HEPES (pH 7.6), 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.05% NaN_3 (FACS buffer) at 4°C. Cells were incubated for 60 minutes (4°C) with mouse anti–PEDF antibody (Chemicon, Hofheim, Germany). The unbound antibody was removed by washing in FACS buffer, and a secondary phycoerythrin-conjugated goat polyclonal anti–mouse IgG (Dianova) was added. After they were washed, the samples were analyzed (FACScan; BD Biosciences, Mountain View, CA). Gates were set to exclude nonviable cells, and histograms were recorded to determine the percentage and mean fluorescence intensity (MFI) of labeled cells defined by scatter gates. Data are presented as histograms, with cell number (*y*-axis) plotted against fluorescence intensity on a logarithmic scale (*x*-axis).

Enzyme-Linked Immunosorbent Assay

Cell culture supernatants were assayed for the presence of PEDF using a sandwich ELISA system purchased from BioProducts. Supernatants were collected from cell culture wells, cleared by centrifugation, and analyzed in duplicate.

Immunoblot Detection

In several experiments culture supernatants were collected after incubation of the cells for 24 hours and were immunoblotted with an anti–PEDF antibody. Briefly, supernatants were treated with electrophoresis sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% sucrose; final concentrations), heated to 100°C for 10 minutes, and run on 12% polyacrylamide gels. The separated proteins were transferred onto a polyvinylidene difluoride membrane, and PEDF was detected using polyclonal anti–PEDF (BioProducts) and an alkaline phosphatase-conjugated goat-anti–rabbit secondary antibody from Cell Signaling Technology (Danvers, MA). Blots were stained using 0.5 mg/mL nitroblue tetrazolium chloride and 0.25 mg/mL 5-bromo-4-chlor-3-indolyl phosphate (Sigma), and PEDF levels were quantified by densitometry and standardized to the respective cell number.

Reverse Transcription, PCR, and Quantitative RT-PCR

Total RNA of cells was prepared using a commercially purchased RNA isolation kit (Qiagen, Hilden, Germany). Contaminating genomic DNA was eliminated with 1 U DNase I (Life Technologies), and single-stranded cDNA was synthesized from 1 μg total RNA in a 20-μL

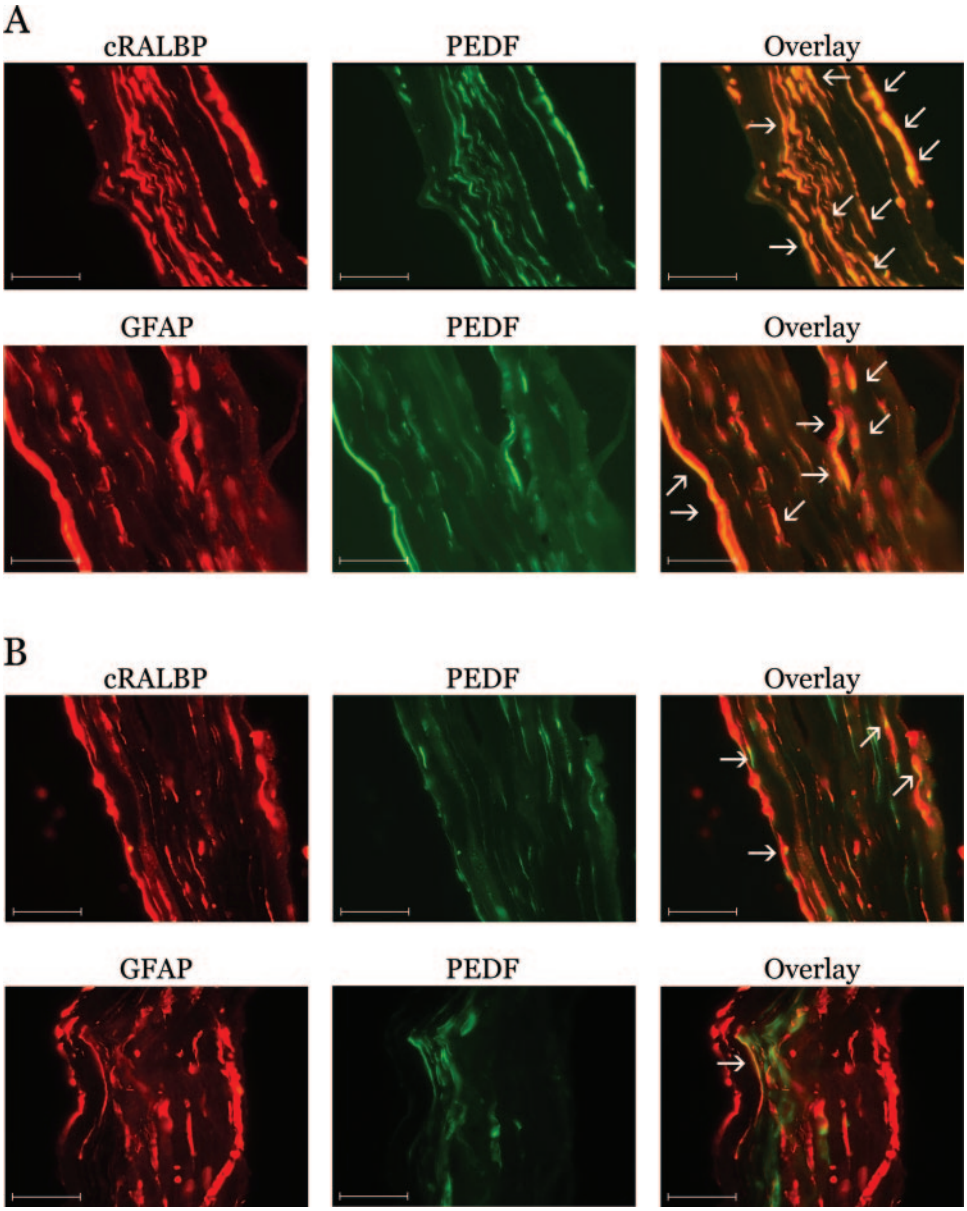

FIGURE 1. PEDF levels are strongly reduced in fibrovascular tissues from patients with PDR. Fibrovascular membranes stained with antibodies against CRALBP, GFAP (*red*), and PEDF (*green*) are shown. Membranes originating from patients with (**A**) choroidal neovascularization caused by pathologic myopia and (**B**) PDR are shown. Negligible immunostaining was found with nonimmune immunoglobulin controls (not shown). *Arrows*: areas with simultaneous immunoreactivity for PEDF and CRALBP or GFAP. Results are representative of four membranes from patients with myopia and PDR. Scale bars, 50 μm.

reaction (200 U Superscript II reverse transcriptase; Life Technologies), 500 μM dNTP, and 0.5 μg oligo(dT)$_{15}$. Real-time PCR used 2 μL cDNA and was performed with a PCR detection system (Single-Color Real-Time; Bio-Rad, Munich, Germany) using 200 nM each of primers 5′ CAT GAC ATA GAC CGA GAA CTG 3′ and 5′ AAG GAT TGC AGC TTC ATC TCC 3′, both specific for human PEDF mRNA (GenBank accession number NM_002615) and 5′ CAC CAC ACC TTC TAC AAT GA 3′ and 5′ CAA ACA TGA TCT GGG TCA TCT 3′, and both specific for human β-actin mRNA (GenBank accession number NM_001101). The PCR mix was denatured at 95°C for 6 minutes, followed by 40 cycles of melting at 95°C for 10 seconds, annealing at 58.5°C for 25 seconds, and elongation at 72°C for 25 seconds. Fluorescence changes were monitored after each cycle. The *C*t (threshold cycle) values were used, and PEDF mRNA levels were normalized to β-actin levels. Quantitation was calculated as follows: mRNA levels (fold change) = $2^{-\Delta\Delta Ct}$, with $\Delta Ct = Ct_{\text{PEDF}} - Ct_{\beta\text{-actin}}$ and $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{medium control}}$.

Statistical Analysis

All values were presented as mean ± SEM unless otherwise stated. Statistical comparison of groups was performed using one-way ANOVA, and $P < 0.05$ was considered significant.

RESULTS

PEDF Levels in Fibrovascular Retinal Tissue in Patients with Diabetic Retinopathy

Previous reports suggested that intraocular PEDF levels decrease in ischemic angiogenic eye diseases, particularly in PDR.[27–31] To determine whether PEDF downregulation occurs in ischemic retinopathies within the proliferative tissue, we investigated surgically excised retinal neovascular membranes originating from patients with PDR and compared PEDF immunoreactivity to that in fibrovascular proliferative retinal tissue from patients with subfoveal choroidal neovascularization caused by pathologic myopia (considered as nonhypoxic control). As detected by double-labeling immunohistochemistry, significant PEDF staining in the control membranes coincided with CRALBP and GFAP immunoreactivity, suggesting that retinal glial (Müller-cell derived, dedifferentiated) cells contribute significantly to PEDF production (Fig. 1A). However, considerably lower PEDF levels were found in the membranes of patients with PDR than in those from donors without diabetes (Fig. 1B). To identify differences between membranes from

patients with diabetes and control membranes, the membranes were examined according to a scoring system (see Materials and Methods). PEDF production in the control membranes was assigned a score of 3.47 ± 0.68 and in the retinal neovascular membranes a score of 1.57 ± 0.23. These results indicate that the proliferation of retinal cells in PDR is associated with downregulated PEDF tissue levels and that PEDF downregulation also involves Müller cell–derived elements in the proliferative tissues.

Roles of Protein Synthesis and Hypoxia in the Control of PEDF Production by Müller Cells

Based on the findings indicated, we studied PEDF turnover under the control of de novo translated proteins and the effects of hypoxia onto PEDF production and release, each with cultured primary retinal glial (Müller) cells.

The first problem was addressed by applying the translational inhibitor CHX. In its presence, elevated rather than decreased levels of PEDF protein were surprisingly determined in the culture media, independent of whether the cells were cultured under normoxia or hypoxia (2.5% O_2; Fig. 2A). We also observed an apparent CHX-mediated stabilization of PEDF at the mRNA level; PEDF mRNA levels increased in normoxia, and PEDF mRNA decline observed under hypoxia (2.5% O_2) failed to occur (Fig. 2D).

Further experiments were performed to compare PEDF protein and mRNA levels representative of primary Müller cells under normoxic and hypoxic conditions, respectively. Moreover, we assessed whether Müller cell–derived PEDF was entirely released into the medium or may be (partially) associated with their cell surface.

When Müller cells were cultured for 24 hours under normoxic, moderately hypoxic (2.5% O_2), or strongly hypoxic conditions (0.2% O_2), PEDF protein release and PEDF mRNA expression varied. However, the oxygen effects demonstrated no simple pattern. PEDF protein levels declined substantially at 2.5% O_2 (1.80- ± 0.58-fold; Figs. 2A, 3A) but declined less at 0.2% O_2 (1.20- ± 0.10-fold; Figs. 2B, 3A). With the use of native Müller cells and flow cytometry, we determined whether this difference might be accounted for by assuming an enhanced cell surface association of the released protein under strong hypoxia. We found PEDF to be significantly associated with the cell surface of Müller cells already under normoxic conditions (Fig. 2C, left). MFI values of PEDF-immunopositive Müller cells (as a measure of PEDF cell surface expression) decreased, rather than increased, on exposure to hypoxia, indicating decreased surface association (Fig. 2C, right, black histogram). Thus, total PEDF production was decreased under hypoxia, even at 0.2% O_2. PEDF mRNA levels showed an even more complex response to hypoxia. We observed a moderate downregulation at 2.5% O_2 (Fig. 2D) but a 3.37-fold upregulation at 0.2% O_2 (± 0.75, $n = 6$; $P < 0.01$; Fig. 2E).

Together these results suggest that regulatory mechanisms at the mRNA and the cotranslational or posttranslational levels may play decisive roles in controlling PEDF production by Müller cells.

Regulation of PEDF Production by Müller Cells at Different Oxygen Concentrations

To investigate whether PEDF expression in Müller cells depends on the severity of hypoxia, the cells were left under normoxia or exposed for 24 hours to 10%, 5%, 2.5%, 1.7%, and 0.2% O_2, respectively. As expected, stronger hypoxia resulted in a progressively increasing VEGF-A release, mounting to approximately 50-fold elevated concentrations at 0.2% O_2 (data not shown). By contrast, a parabolic oxygen concentration-dependent release curve was observed for PEDF. Figure 3A shows that PEDF protein levels in the culture media decreased

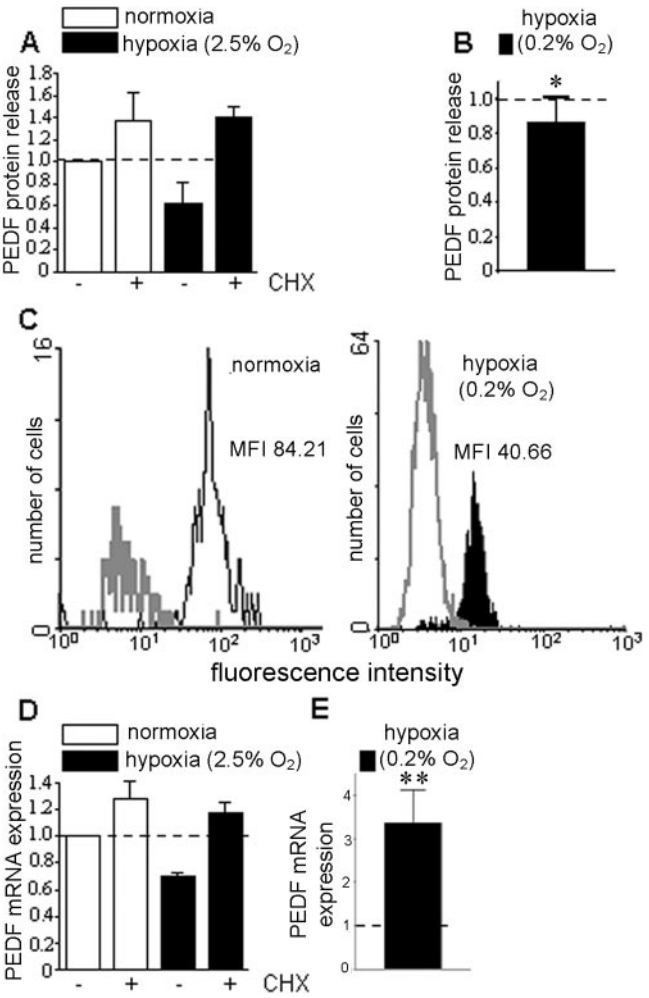

Figure 2. Stabilization of PEDF production by Müller cells induced by the protein synthesis inhibitor CHX and hypoxia-induced regulation of PEDF in primary guinea pig Müller cells. Cells were exposed to either CHX or hypoxia, and PEDF expression was investigated at the protein and the mRNA levels after 24 hours of cell culture. Data from two single experiments involving the effect of CHX were averaged; error bars represent SD. Relative PEDF mRNA expression was quantified by real-time PCR. All bars indicate changes in PEDF levels in relationship to levels measured using control cultures (normoxic conditions without additives). (**A**) Under normoxic and hypoxic conditions, CHX exposure of cells leads to an increase of PEDF protein release. (**B**) Strong hypoxia (0.2% O_2) induces decreased PEDF protein release from Müller cells ($n = 8$; $^*P < 0.05$). Media from hypoxia-treated or control Müller cell cultures were immunoblotted with an antibody specific for PEDF, and the bands were semiquantified by densitometry and normalized to the cell number. (**C**) PEDF is exposed on the cell surfaces of Müller cells, demonstrating a lower presence in hypoxia (0.2% O_2). Anti-PEDF–stained Müller cells (*gray lines* in both panels represent mouse IgG2a isotype control) were analyzed by immunofluorescence staining and flow cytometry. As a measure of cell surface expression levels, MFI of $PEDF^+$ cells is indicated. Results are representative of three experiments. (**D**) Elevated PEDF mRNA levels under normoxic conditions and impaired downregulation under hypoxia (2.5% O_2) in CHX-exposed Müller cells. (**E**) Hypoxia (0.2% O_2) leads to increasing PEDF mRNA expression ($n = 6$; $^{**}P < 0.01$).

to a minimum (approximately 50% of normoxic levels) under relative mild hypoxia (2.5%–10% O_2) but increased again under stronger hypoxia (1.7% O_2) almost to control levels (0.2% O_2).

PEDF mRNA demonstrated a similar nonlinear correlation to the O_2 concentration (Fig. 3B); there was even an increase of PEDF mRNA observed at the lowest O_2 concentration tested

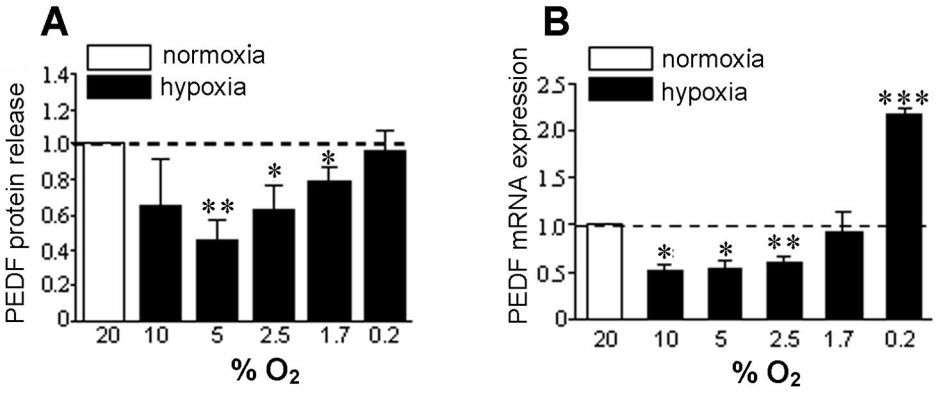

FIGURE 3. PEDF production by Müller cells under different degrees of hypoxia. PEDF protein secretion was determined by ELISA, and mRNA levels were measured by real-time PCR. Results from three to seven experiments performed on cells from different animals are given ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$).

(Fig. 3B). After 40 hours of culture under hypoxic conditions, PEDF levels tended to increase, demonstrating an upregulation at both the mRNA and the protein levels at 2.5%, 1.7%, and 0.2% O_2 (data not shown).

DISCUSSION

Our study was aimed at elucidating the role of the Müller cell in ischemic eye diseases such as PDR by assessing the O_2-dependent regulation of Müller cell–derived PEDF as an important antiangiogenic and neurotrophic/neuroprotective factor. Although positive regulators of angiogenesis such as VEGF-A are present in the healthy adult eye,[43] active angiogenesis does not occur, most likely because of the predominant activity of antiangiogenic molecules such as PEDF. Indeed, PEDF can counterbalance proangiogenic conditions in retinal ischemia and PDR.[7,8,11,26] We have previously demonstrated that Müller cells express and release PEDF[10] and that Müller cells provide a permanent antiproliferative condition for vascular endothelial cells in the healthy retina by releasing antiangiogenic factors such as PEDF, TGF-β2, and thrombospondin-1.[44]

In ischemic retinopathies, however, VEGF-A is upregulated while PEDF levels decrease.[14,27–31] We report here that PEDF levels are low in epiretinal membranes originating from patients with PDR and that PEDF immunoreactivity partially coincides with CRALBP- and GFAP-positive structures (with Müller cell–derived elements) in the fibrovascular membranes. We conclude that Müller glial cells that dedifferentiate and migrate to and from preretinal tissues in PDR[45] simultaneously downregulate their PEDF release. Such a view is consistent with earlier immunohistochemical data demonstrating levels of intraretinal PEDF to be low or not detectable in the eyes of patients with PDR.[27] We consider low PEDF levels in PDR to be related to poor PEDF production by Müller cells, which may thus contribute to perpetuating active proliferation of retinal endothelial cells. Together with the fact that PEDF levels are reduced in the ocular fluid of patients with PDR, our findings lend support to the commonly accepted concept that a disturbed balance between proangiogenic and antiangiogenic mediators accounts for pathologic retinal angiogenesis and that intraretinal PEDF levels are inversely correlated to degree of retinal neovascularization.[26] Müller cells may thus be crucially involved in the pathophysiology of retinal ischemia.[46]

Another important activity of Müller cells accompanying ischemic injury, however, is related to their ability to protect photoreceptor cells from cell death. Neuronal cell death from apoptosis is an important and potentially sight-threatening complication of retinal damage or injury.[47] In such a scenario, Müller cells produce neurotrophic factors such as basic fibroblast growth factor, ciliary neurotrophic factor, and VEGF-A, which directly act on photoreceptors.[48–51] Given that various studies have supported a role of PEDF in neuronal survival, especially the survival of retinal neurons,[2–6] Müller cell–derived PEDF might supply another important neuroprotective activity in ischemic injury[4,5] and may even prevent a degeneration-accelerating response.[52]

The regulation of PEDF in the retina is not fully understood, which is in marked contrast to that of its apparent opponent, VEGF-A. Previous observations suggest that different regulatory mechanisms may control the production of PEDF under hypoxia in different cell types. For example, we have demonstrated mild hypoxia (2.5% O_2) to be associated with downregulated PEDF mRNA and protein levels in a Müller cell line (MIO-M1).[10] PEDF mRNA and protein were also downregulated in the ischemic retina, as shown in a rat model of retinal neovascularization.[26] However, in retinoblastoma and RPE cells, PEDF expression was reported to decline at the protein level by hypoxia (0.5% O_2), with no significant change in PEDF mRNA.[7,53] Thus, a hypoxia-dependent regulation of PEDF expression may occur at both the level of transcription and the level of mRNA stability. Furthermore, the release of PEDF from Müller cells may not depend on intracellular PEDF stores or concentrations in a linear manner.

Additionally, there appears to be a further level of regulation, relying on the accessibility of cell-bound PEDF. Although it is known that charged surfaces of the PEDF molecule interact with proteins of the extracellular matrix, we have demonstrated here for the first time a possible association of PEDF with the cell surface. In particular, PEDF binds to glycosaminoglycans such as heparin and chondroitin sulfates,[54] which are known to be present on the cell surface. This binding is reminiscent of that of several other biologically active factors including VEGF-A, fibroblast growth factors, thrombospondins, and chemokines, all of which are known to interact with heparin and heparan sulfate glycosaminoglycans. Cell surface–anchored PEDF provides the possibility of sequestration and controlled release and may allow direction of the biological effects of PEDF toward neighboring cells.

We show here for the first time that protein factors, the de novo synthesis of which is blocked by CHX treatment, contribute to PEDF levels produced by Müller cells. Such a contribution apparently limits PEDF expression in the cell, ensuring the possibility of downregulation, for example, under mild hypoxia (Figs. 2A, 2D, 3).

We have here analyzed for the first time how PEDF mRNA levels and protein secretion depend on different degrees of hypoxia. We show that PEDF mRNA levels and protein release are largely dependent on the degree of hypoxia over a wide range of O_2 levels. Although PEDF mRNA and protein levels were decreased at 10% to 2.5% O_2, the PEDF decline regressed at lower oxygen concentrations (1.7% O_2) and finally (at 0.2% O_2) even turned to increasing PEDF expression at the mRNA level. This might have resulted from the presence of more than one regulatory mechanism whose predominance changed in

depending on the degree of hypoxia and gave rise to a differential modulation of PEDF mRNA expression and protein secretion. Obviously, not only regulation of the mRNA level but also posttranslational mechanisms occur that play decisive roles in controlling PEDF release from Müller cells.

Our results may be explained by the assumptions that the downregulation of PEDF expression, such as under hypoxia, involves the de novo synthesis of a hitherto unknown protein factors (because PEDF mRNA levels increase, rather than decrease, under CHX; Fig. 2D), and that Müller cells contain excessive stores of PEDF protein (because its release is even enhanced under CHX; Fig. 2A), but these assumptions remain to be proven.

To summarize, we show here for the first time that PEDF production by Müller cells obeys an O_2-dependent biphasic curve involving a decrease of PEDF mRNA during mild hypoxia but an increase of PEDF mRNA under strong hypoxia (Fig. 2C). These results suggest that in mild hypoxia with moderately upregulated VEGF-A levels, PEDF is downregulated to provide angiogenesis-permissive conditions; however, when the tissue supply with oxygen and nutrients becomes severely compromised in ischemia and hypoxia that is strong (reflected in our experiments at 0.2% O_2) or prolonged (reflected in our experiments >24 hours; data not shown), elevated levels of PEDF may ensure its cell-preserving function (because of the neurotrophic/neuroprotective properties), thus facilitating retinal neuronal and glial cell survival. The administration of PEDF in animal models was previously reported to increase the survival of retinal neurons in ischemia.[4,5] Thus, we propose that retinal PEDF is charged with the task of balancing a state of angiogenic quiescence against the need to maintain the protection of retinal neuronal and glial cells.

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