Pigment Epithelium-Derived Factor Binding to VEGFR-1 (Flt-1) Increases the Survival of Retinal Neurons

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Purpose. The purpose of this study was to examine possible involvement of vascular endothelial growth factor (VEGF) receptor (VEGFR)-1/Flt-1 in pigment epithelium-derived factor (PEDF)-promoted survival of retinal neurons.

Methods. Survival of growth factor-deprived retinal ganglion cells (RGCs) and R28 cells and activation of ERK-1/-2 MAP kinases were assessed in the presence of PEDF, placental growth factor (PlGF), and VEGF using cell cultures, viability assays and quantitation of ERK-1/-2 phosphorylation. VEGFR-1/Flt-1 expression was determined using quantitative PCR (qPCR) and Western blotting. VEGFR-1/Flt-1 was knocked down in R28 cells by small interfering RNA (siRNA). Binding of a PEDF-IgG Fc fusion protein (PEDF-Fc) to retinal neurons, immobilized VEGFR-1/Flt-1 and VEGFR-1/Flt-1-derived peptides was studied using binding assays and peptide scanning.

Results. PEDF in combination with PlGF stimulated increased cell survival and ERK-1/-2 MAP kinase activation compared to effects of either factor alone. VEGFR-1/Flt-1 expression in RGCs and R28 cells was significantly upregulated by hypoxia, VEGF, and PEDF. VEGFR-1/Flt-1 ligands (VEGF and PlGF) or soluble VEGFR-1 (sflt-1) competed with PEDF-Fc for binding to R28 cells. Depleting R28 cells of VEGFR-1/Flt-1 resulted in reduced PEDF-Fc binding when comparing VEGFR-1/Flt-1 siRNA- and control siRNA-treated cells. PEDF-Fc interacted with immobilized sflt-1, which was specifically blocked by VEGF and PlGF. PEDF-Fc binding sites were mapped to VEGFR-1/Flt-1 extracellular domains D3 and D4. Peptides corresponding to D3 and D4 specifically inhibited PEDF-Fc binding to R28 cells. These peptides and sflt-1 significantly inhibited PEDF-promoted survival of R28 cells.

Conclusions. These results suggest that PEDF can target VEGFR-1/Flt-1 and this interaction plays a significant role in PEDF-mediated neuroprotection in the retina.

Keywords: retinal degeneration, neuroprotection, pigment epithelium-derived factor (PEDF), vascular endothelial growth factor (VEGF) receptor

Neurodegenerative diseases of the retina are a primary cause of visual impairment and irreversible blindness worldwide.[1] Among these diseases are glaucoma, and diabetic and ischemic retinopathies, which exhibit a characteristic feature, the loss of retinal ganglion cells (RGCs) by apoptosis.[2–5] Glaucoma refers to a group of degenerative eye diseases characterized by progressive RGC demise and optic nerve damage, often due to intraocular pressure elevation. Neurodegenerative changes in the inner retina also occur in diabetic retinopathy (DR), where the apoptotic demise of neurons such as RGCs may precede retinal microangiopathy, retinal ischemia, and macular edema.[6,7] Whereas neuronal viability is compromised in retinal neurodegenerative diseases, giving rise to degeneration and loss of RGCs,[8] there are trophic factors (neurotrophins) that counteract apoptosis.[9] However, in spite of the effectiveness of neuroprotective mediators in a number of experimental approaches, there is currently no neuroprotective/ neuroregenerative therapy available for treating chronic retinal neurodegenerative processes.

Pigment epithelium-derived factor (PEDF) is a multifunctional secreted protein, which has been shown to promote the survival of retinal neurons under several pathological conditions[10–18] (reviewed in Ref. 19). Because PEDF also inhibits inflammation, vascular hyperpermeability, and angiogenesis in ischemic retinopathies[20,21] it has emerged as an ideal candidate molecule for the therapeutic management of retinal diseases. Vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) are related dimeric glycoproteins and members of the VEGF

protein family. VEGF-A (hereafter referred to as VEGF) is an important neuroprotective mediator in the retina.[22–24] PlGF protects retinal pigment epithelial (RPE) cells against apoptosis[25] and it is likely that this factor also contributes to viability maintenance of retinal neurons.[26] Both cytokines, VEGF and PlGF, interact with VEGF receptor (VEGFR)-1/Flt-1, a high-affinity receptor tyrosine kinase that upon ligand binding undergoes autophosphorylation and transmits ligand-specific signals into the cell. In contrast to VEGF, PlGF does not interact with VEGFR-2/KDR,[27] which displays a lower affinity for VEGF but higher tyrosine kinase activity compared to VEGFR-1/Flt-1. Whereas VEGF-mediated neuroprotection (similar as VEGF-induced angiogenesis) is based on activation of VEGFR-2/KDR,[22–24,28,29] PlGF exerts effects exclusively through VEGFR-1/Flt-1, thereby inducing signaling and DNA synthesis in endothelial cells[30] and protecting survival of retinal neurons.[26]

The neuroprotective effects of PEDF may occur in a pathologically altered retinal milieu that contains elevated levels of VEGF and PlGF, in particular, under ischemic/hypoxic conditions.[31–33] Because PlGF, opposite to VEGF, binds to one distinct receptor the present study has focused on the role of PlGF and VEGFR-1/Flt-1, seeking to explore whether PlGF stimulates RGC survival by synergizing with PEDF. We have explored the idea that PEDF is bound by VEGFR-1/Flt-1, thereby acting as a potential receptor for PEDF. To this end, we have investigated RGCs and R28 cells, where we identified PEDF binding sites in VEGFR-1/Flt-1. It is therefore likely that PEDF-VEGFR-1/Flt-1 interaction plays a role in promoting the survival of retinal neurons.

Materials and Methods

Culture and Stimulation of Cells

Cells were routinely cultured at 37°C, 5% CO_2, 95% air, or under hypoxia (0.2% O_2 and 5% CO_2) in media supplemented with 100 U/mL penicillin/ 100 µg/mL streptomycin. Identity of retinal progenitor (R28) cells[34,35] (a gift of Dr. Katharina Bell, Department of Ophthalmology, Johannes Gutenberg University, Mainz, Germany) was confirmed by immunofluorescence staining of RGC-associated molecules (CD90/ Thy-1, neurofilament H) and glial fibrillary acidic protein. R28 cells were cultured in Dulbecco's minimal essential medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with/ 10% fetal calf serum (FCS). Procedures for RGC isolation from mouse retinae were approved by regulatory authorities (Landesdirektion Sachsen and Leipzig University Medical Faculty). RGCs were prepared from retinae of 7 day old mice by immunopanning, as previously described.[10] Cell-based assays were performed in neurobasal medium (RGCs) or DMEM (R28 cells) in the absence of serum and growth factors. Where appropriate, R28 cells were starved overnight in DMEM. RGCs and R28 cells were left untreated or were treated with recombinant rat VEGF-164 (50 ng/mL; Biolegend, Koblenz, Germany), human PlGF-2 (50 ng/mL; Biotechne, Wiesbaden, Germany), and human PEDF (500 ng/mL; Biomol, Hamburg, Germany), either for 30 minutes (ERK-1/-2 MAP kinase activation analyses) or 24 hours (VEGFR-1/Flt-1 expression analyses). For quantitative PCR (qPCR) and Western blotting analyses, the cells were washed with ice-cold PBS and extracted in appropriate lysis buffers.

Cell Survival Assays

Neuronal cells were cultured for 24 hours in the presence or absence of rat PlGF-1 (50 ng/mL; PeproTech, Hamburg, Germany: stimulation of RGCs), human PlGF-2 (stimulation of R28 cells), human PEDF, rat VEGF-164 (100 ng/mL), or a combination of factors. Where appropriate, cells were incubated in the presence of a sflt-1 protein consisting of soluble VEGFR-1/Flt-1 and the Fc part of human IgG1 (#321-FL, Biotechne). Survival of RGCs was determined by a live-dead assay using calcein acetoxymethyl ester (Calcein-AM; Molecular Probes, Inc., Eugene, OR, USA; 2 µM) as described previously.[10] Alternatively, R28 cells were exposed to human PEDF (500 ng/mL) after preincubation (2 hours at 37°C) with sflt-1, human control IgG (each at 50 ng/mL) or peptides (see below; 10 µM). To determine the survival of metabolically viable cells, a solution of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT; final concentration 0.1 mg/mL) was added 20 hours after setting up the cultures. Four hours later, the cells were solubilized with DMSO (100 µL), and reduction of MTT was determined at 570 nm.

Western Blotting

Lysates of R28 cells were separated by SDS-PAGE and proteins were transferred to a PVDF membrane. Immunoblots were processed as described previously.[36] VEGFR-1/Flt-1 expression was analyzed using a rabbit anti-Flt-1 antibody (#sc-9029; Santa Cruz Biotechnology, Heidelberg, Germany; 4 µg/mL). β-actin, serving as a loading control, was detected with a rabbit anti-β-actin mAb (D6A8; Cell Signaling Technology, Danvers, MA, USA). Polyclonal antibody #sc-9029 was validated using cells transfected with short interfering RNA (siRNA; Supplementary Fig. S1). To detect ERK-1/-2 phosphorylation, the blots were probed with a phospho-specific rabbit anti-p44/p42 ERK-1/-2 (Thr202/ Tyr204) antibody and, in parallel, an antibody directed against ERK-1/-2 (both from Cell Signaling Technology). In other experiments, sflt-1 was run by SDS-PAGE (400 ng per lane) and transferred to nitrocellulose. Blots were blocked and, where applicable, preincubated with human PEDF (500 ng/mL). Strips of blots were probed with a mouse anti-human IgG Fc mAb (Biolegend) or PEDF-Fc and then exposed to alkaline phosphatase-conjugated goat anti-mouse IgG (Dianova).

Reverse Transcription, PCR, and Quantitative RT-PCR

Total RNA from cells was prepared, treated with DNase I (Invitrogen) and subjected to reverse transcription and PCR using standard procedures. Aliquots of cDNA (2 µL) and primers (200 nM) were treated with a Maxima SYBR Green mastermix (Thermo Fisher Scientific) and amplified DNA was visualized using agarose gel electrophoresis and ethidium bromide staining. For qPCR analyses, cDNAs were amplified at comparable efficiencies using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), in the presence of primers (200 nM; Supplementary Table S1). Relative VEGFR-1/Flt-1 expression levels were obtained by calculating $2^{-\Delta\Delta Cq}$ (fold expression), with ΔCq (quantification cycle) corresponding to VEGFR-1/Flt-1 expression normalized to β-actin expression ($C_{q,\ \text{VEGFR-1/Flt-1}} - C_{q,\ \beta\text{-actin}}$), and where $\Delta\Delta C_q$ was calculated by subtracting sample ΔC_q values of exper-

imental from ΔC_q values of control conditions. If not indicated otherwise, means ± SEM of data from independent cell culture experiments are shown, each analyzed at least in triplicate.

Construction of Mouse IgG Fc Fusion Proteins

Overexpression plasmids were generated by cloning into pcDNA3.1 (+) neo (Invitrogen). In brief, a PEDF-encoding cDNA derived from human RPE cells was placed upstream of a DNA sequence encoding for a mouse IgG2b Fc fragment and a *C*-terminal consensus biotinylation peptide sequence (DPNSGSLHHILDAQKMVWNHR) and ligated with the plasmid using *Kpn* I – *Eco*R I restriction endonuclease cutting sites. A similar plasmid encoding for the extracellular part of ADGRE5/CD97 fused to mouse IgG2b (used for control purposes) was a kind gift from Dr. Jörg Hamann (Academic Medical Center, Amsterdam, The Netherlands). HEK-293 cells (American Type Culture Collection, Rockville, MD, USA) were transfected with the plasmids using calcium phosphate precipitation. Cell-free supernatants collected 36 hours after transfection contained soluble proteins fused to mouse IgG2b Fc region (hinge-Cγ2-Cγ3), accessible to biotinylation by *E. coli* biotin holoenzyme synthetase BirA. Fusion proteins (PEDF-Fc and control fusion protein) were purified using protein A sepharose CL4B (Pharmacia, Uppsala, Sweden) and, where applicable, subjected to biotinylation.[37]

Immunofluorescence Staining of Retinae and Cells

Use of human retinal tissue was in accordance with the Declaration of Helsinki and approved by the Leipzig University (Medical Faculty) ethics committee. Retinae from two donors without known histories of retinal disease were included in immunofluorescence staining. The number of experiments performed with RGCs from different mice or independently cultured R28 cells are indicated in the appropriate figure legends. Paraformaldehyde (PFA)-fixed retinal sections and cells were permeabilized/ blocked (2 hours) using PBS/ 0.3% Triton X-100, 1% DMSO, 5% normal goat serum at room temperature. Specimens were incubated overnight (4°C) with antibodies directed against VEGFR-1/Flt-1 (mAb #MAB321, Biotechne, for retinae or #sc-9029 for cells). Double staining of retinae included rabbit anti-class III β-tubulin (TUJ1; Covance, Princeton, NJ, USA). Alternatively, PEDF-Fc or fusion protein control (each approximately 50 µg/mL) were applied to label cells, where indicated, after preincubation overnight (4°C) with 500 ng/mL human PEDF (Biomol), sflt-1 (Biotechne), or human control IgG (Sigma-Aldrich; each at 1 µg/mL). Specimens were washed 3 times and incubated with Cy2-or Cy3-conjugated goat anti-mouse IgG (Dianova) for 2 hours. Expression of βIII-tubulin or VEGFR-1/Flt-1 in cells was revealed using Cy3-conjugated goat anti-rabbit IgG (Dianova). Where applicable, cells were washed, counterstained with DAPI, and mounted.

Binding Assays

R28 cells were fixed in PBS/ 1% PFA for 30 minutes on ice and permeabilized (see preceding paragraph), followed by incubation in binding buffer (TBS/ 5% BSA, 1 mM $MgCl_2$, and 1 mM $CaCl_2$) for 30 minutes. Where applicable, the cells were preincubated with human PEDF (500 ng/mL), rat VEGF (100 ng/mL), and human PlGF-2 (50 ng/mL), or PEDF-Fc was preincubated with peptides, sflt-1, or human IgG control for 2 hours at 37°C. PEDF-Fc, in the presence or absence of competing reagents, and appropriate controls (mouse IgG2b and fusion protein control) were added to cells (4°C, overnight). Cells were washed three times with binding buffer and processed for colorimetric immunodetection as described for Western blots.[36] The colored precipitate was solubilized in DMSO, and degree of binding was quantitated using a spectrophotometer.

In a solid phase binding assay, sflt-1 was immobilized by consecutively coating wells of a 96-well plate with anti-VEGFR-1/Flt-1 (#MAB321, 1 µg/mL) and sflt-1 (0.5 µg/mL, in PBS, 2% BSA) at 4°C overnight. After washing with PBS/ 2% BSA, the wells were blocked and biotinylated PEDF-Fc (1 µg/mL, in PBS, 2% BSA) was added in the absence or presence of human PlGF-2 or human VEGF (Biotechne) as indicated in Figure 5. The wells were washed 3 times with binding buffer and binding of biotinylated PEDF-Fc was quantitated using horseradish peroxidase-conjugated streptavidin (Dianova) and 3, 3′, 5, 5′-tetramethylbenzidine (Sigma-Aldrich) as a substrate.

Gene Expression Silencing by siRNA

VEGFR-1/Flt-1 was depleted from R28 cells using RNA interference. Briefly, 75 pmol of rat VEGFR-1/Flt-1-targeting siRNAs (Supplementary Table S2) and non-targeting siRNA (Santa Cruz; Cat# sc-37007; negative control) were complexed with Lipofectamine RNAiMAX and added to cells in 6-well plates in antibiotic-free Opti-MEM (Thermo Fisher Scientific).

Peptide Synthesis and Scanning

Single soluble peptides were synthesized using standard N-(fluorenyl-9-methoxycarbonyl [Fmoc]) chemistry and purified using reverse-phase HPLC. Purity (95%) was confirmed by mass spectrometry. The synthetic heptadecapeptides, AE10, AF10, DX21, and DW21 corresponding to amino acid sequences in the extracellular portion of VEGFR-1/Flt-1 (GenBank accession number NP_002010.2), and an irrelevant control peptide (amino acid sequence: FYIPTGSESNTPIMLQLTKEGRD) were applied in assays with PEDF-Fc.

A peptide library of soluble VEGFR-1/Flt-1 preprotein (GenBank accession number NP_001153392.1) was produced using the SPOT peptide technology.[38] Briefly, overlapping tridecapeptides (11 amino acids overlap) were synthesized on a cellulose membrane (Whatman 50) in an arrangement of 27 columns and 13 rows. The membrane was probed with fusion proteins at 4°C overnight and binding of PEDF-Fc was detected by incubation with peroxidase-linked goat-anti mouse IgG (Dianova) dissolved in TBS/ 0.05% Tween-20/ 2% BSA. The membrane was washed and chemiluminescent spots were produced and recorded using an ECL system (Pierce; Thermo Scientific).

Statistics

Values are given as means ± SEM of data from independent cell culture experiments or binding assays. For cell survival, ERK-1/-2 MAP kinase, qPCR, and binding assays the number (*n*) of biological replicates is indicated in the figure legends. Standard 1-way ANOVA followed by Tukey's post hoc test

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was used for multiple-group comparisons. Single comparisons were made using one sample *t*-test when data were normalized relative to control. Statistical significance was accepted if $P < 0.05$.

Results

PlGF Potentiates PEDF-Stimulated Survival of Retinal Neurons

We have previously characterized PEDF as a survival-promoting factor for RGCs in vitro, in cellular interactions with retinal (glial) Müller cells, which release several neuroprotective mediators.[10,11] VEGF[22–24] and PlGF[26] may modulate PEDF-mediated effects in neuronal cells, because both cytokines exert effects on neuronal survival. We have exposed 24-hour cultures of primary RGCs and R28 cells in growth factor-deprived medium to recombinant factors, that is, human PEDF combined with rat VEGF, rat PlGF (RGCs), or human PlGF (R28 cells) and studied their effects on cell survival and ERK-1/-2 MAP kinase activation. When stimulated by PlGF, the survival of cells demonstrated a tendency to increase (RGCs; Fig. 1A) or significantly increased in experiments with R28 cells ($P < 0.05$; Fig. 1C). VEGF significantly promoted the survival of RGCs ($P < 0.01$) and R28 cells ($P < 0.05$; Figs. 1B, 1C). Treating RGCs with both PEDF and PlGF promoted their survival more effectively than either factor alone suggesting a pro-survival benefit with a combination of both mediators (see Fig. 1A). This was confirmed by cultures of R28 cells, where PEDF and PlGF promoted cell survival synergistically, leading to a significantly ($P < 0.05$) increased viability compared to cultures in the presence of either factor alone (see Fig. 1C). However, combined PEDF and VEGF treatment was not superior to individual factors in boosting neuronal survival (see Fig. 1B) and, in R28 cells, a combination of PEDF and VEGF led to an attenuated cell survival ($P < 0.001$; see Fig. 1C).

Activation of ERK-1/-2 MAP kinases has been revealed as a mechanism to transmit PEDF-induced signals in retinal endothelial cells,[39,40] but ERK-1/-2 activation seems also to be involved in PEDF-mediated pro-survival effects on RGCs.[14] To investigate neuroprotective PEDF signaling in the presence of VEGF family members, R28 cells were exposed to PEDF and either PlGF or VEGF, and the status of ERK-1/-2 activation was determined. We found that individually applied factors induced significantly ($P < 0.05$ to $P < 0.01$) increased ERK-1/-2 phosphorylation, and ERK-1/-2 activation was elevated after combined PEDF and PlGF treatment when compared to treatment with PEDF ($P < 0.001$) or PlGF ($P < 0.05$) alone. In contrast, co-stimulation of R28

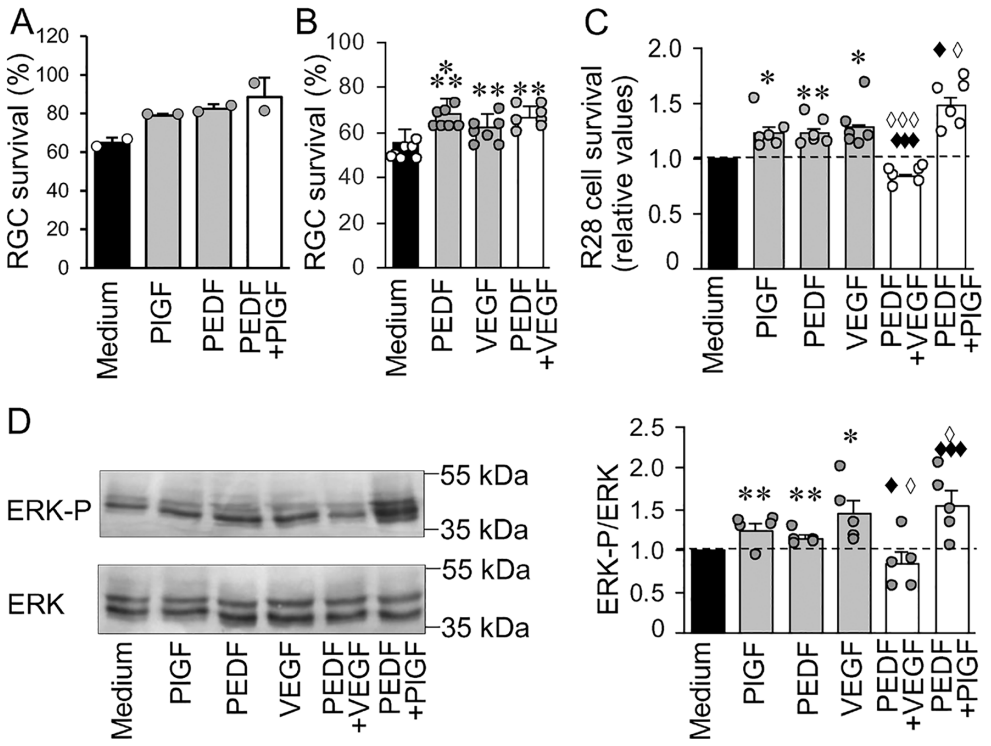

Figure 1. PEDF, PlGF, and VEGF stimulate survival of retinal neurons. Cells were deprived of trophic factors and incubated for 24 hours in the presence or absence of PlGF (50 ng/mL), PEDF (500 ng/mL), and VEGF (100 ng/mL). Shown is the treatment of RGCs and R28 cells with single and combined (*white bars*) factors. (**A**, **B**) The numbers of Calcein-AM-metabolizing RGCs were counted and cell survival was expressed in relationship to numbers of DAPI-positive nuclei: treatment with (**A**) PEDF and PlGF, the panel shows averaged data from two single experiments, error bars depict standard deviation; and (**B**) PEDF and VEGF ($n = 7$; means ± SEM, versus medium control culture, $^{**}P < 0.01$, $^{***}P < 0.001$). (**C**) Survival of stimulated R28 cells was determined by an MTT assay ($n = 6$). (**D**) ERK-1/-2 MAP kinase activation is involved in PEDF-, PlGF-, and VEGF-activated intracellular signaling in R28 cells. Cells were stimulated for 30 minutes, lysed and an equal amount of protein was separated by SDS-PAGE. The figure shows one Western blot out of four independent experiments. In bar charts, columns represent the mean ratio of phosphorylated ERK (ERK-P)/ERK signal intensity ($n = 5$). Results in (**C**) and (**D**, bar charts) are expressed as relative values (means ± SEM, versus medium control culture, *dashed lines*, $^{*}P < 0.05$, $^{**}P < 0.01$; versus PEDF-exposed culture, $^{\blacklozenge}P < 0.05$, $^{\blacklozenge\blacklozenge\blacklozenge}P < 0.001$; versus PlGF-exposed culture, $^{\lozenge}P < 0.05$; versus VEGF-exposed culture, $^{\lozenge}P < 0.05$, $^{\lozenge\lozenge\lozenge}P < 0.001$).

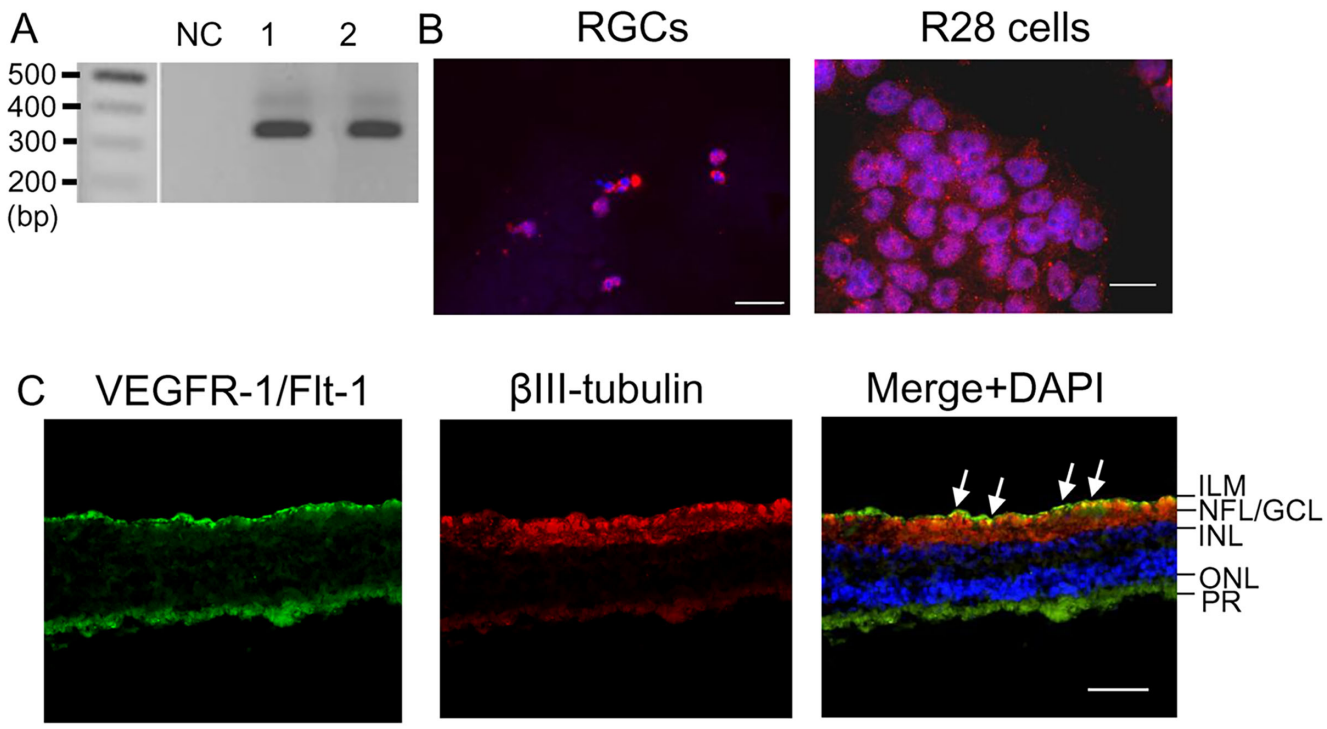

Figure 2. VEGFR-1/Flt-1 expression in retinal neurons demonstrated by (**A**) RT-PCR or (**B**) microscopic imaging following immunofluorescence labeling of RGCs and R28 cells (scale bars = 20 µm) and (**C**) human retina (scale bar = 50 µm). (**A**) RGCs and R28 cells express VEGFR-1/Flt-1 mRNA transcripts. Total RNA was extracted and mRNA expression in (1) RGCs and (2) R28 cells was detected by RT-PCR (NC, negative PCR control). Amplified DNA was visualized using agarose gel electrophoresis. (**B, C**) Labeling of VEGFR-1/Flt-1 with specific antibodies (**B**, rabbit anti-VEGFR-1/Flt-1, *red*; **C**, mAb #MAB321, *green*) and counterstaining of cell nuclei with DAPI (*blue*) were performed using fixed/ permeabilized cells or retinal tissue. Negative controls using rabbit IgG or non-immune mouse IgG1, respectively, demonstrated negligible reactivity (not shown). Images are representative of four independent experiments (**B**) or staining of retinae from two donors (**C**). (**C**) Double-labeling of retinal cryosections revealed co-localization of VEGFR-1/Flt-1 and class III β-tubulin (*red*) in the GCL/NFL (*yellow merge signal*, *arrows*). VEGFR-1/Flt-1 was predominantly localized to the ILM. ILM, internal limiting membrane; NFL, nerve fiber layer; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptor layer.

cells with both PEDF and VEGF significantly ($P < 0.05$) attenuated activation of ERK-1/-2 MAP kinases compared to stimulation with PEDF or VEGF alone (Fig. 1D). These findings raised the possibility that PlGF is able to potentiate PEDF-stimulated ERK-1/-2 signaling in retinal neuronal cells, whereas there is no synergistic effect of VEGF co-stimulation on PEDF-stimulated ERK-1/-2 activation.

Retinal Ganglion Cells and R28 Cells Express and Regulate VEGFR-1/Flt-1

Because VEGFR-1/Flt-1 is the cognate receptor for PlGF[27] it is conceivable that a synergistically PEDF-/PlGF-promoted neuronal survival is regulated by signaling through this receptor. Previous experiments with retinal endothelial cells revealed that PEDF can inhibit VEGF-induced VEGFR-1/Flt-1 autophosphorylation and regulates translocation of its *C*-terminal intracellular domain following proteolytic cleavage.[41] This observation and further previous findings suggesting that PEDF can compete with VEGF for binding to VEGFR-2/KDR[40] prompted us to examine whether PEDF binds to VEGFR-1/Flt-1.

First, we demonstrated that RGCs and R28 express VEGFR-1/Flt-1 at the mRNA level (see Fig. 2A). We confirmed that both cell types express VEGFR-1/Flt-1 using immunofluorescence staining (Fig. 2B). In cryosections of retinae, VEGFR-1/Flt-1 was labeled in some neurons of the ganglion cell layer, as confirmed by double staining of VEGFR-1/Flt-1 and βIII-tubulin, a marker for neurons in the retina. However, VEGFR-1/Flt-1 labeling was most intensely detected in the internal limiting membrane (Fig. 2C). In accordance with previous investigations in RPE cells,[42] we then found that hypoxia (0.2% O_2), a leading pathological condition in retinal neurodegenerative diseases, significantly ($P < 0.05$ to $P < 0.001$) increased VEGFR-1/Flt-1 expression in RGCs (Fig. 3A) and R28 cells (see Figs. 3A, 3B). Compared to normoxic control cultures, VEGFR-1/Flt-1 mRNA and protein levels increased substantially, that is, approximately 1.75-fold in R28 cells at the protein level (see Fig. 3B). To consider a milieu in the retina and vitreous of patients, which is characteristic of ischemic and degenerative diseases and implicates altered levels of PEDF,[43,44] and VEGF,[45,46] we used both survival factors for stimulating retinal neurons. Recombinant PEDF and VEGF significantly ($P < 0.05$ to $P < 0.01$) upregulated VEGFR-1/Flt-1 expression (Figs. 3C, 3D), as reflected by approximately 1.25-fold increased protein levels (see Fig. 3D). These findings indicate that RGCs are a cellular target for the VEGFR-1/Flt-1 cognate ligands, PlGF and VEGF, and suggest that VEGFR-1/Flt-1 levels may undergo regulatory changes in several pathological conditions in the retina, such as hypoxia/ ischemia.

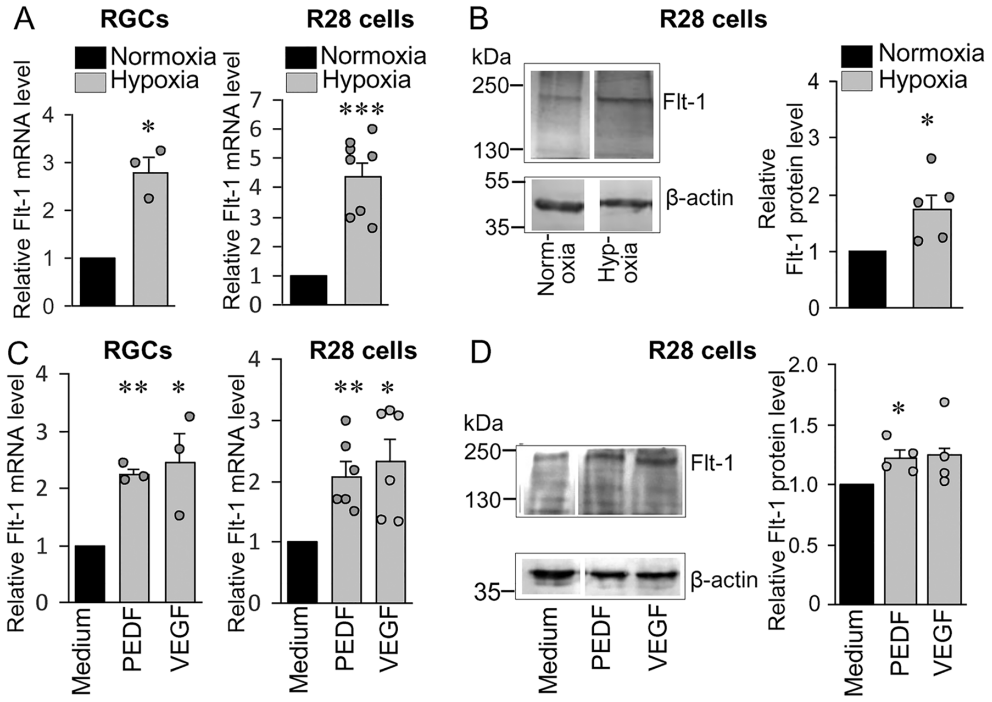

Figure 3. Hypoxia (0.2% O_2), PEDF, and VEGF upregulate VEGFR-1/Flt-1 expression in retinal neurons. (**A**) Elevated VEGFR-1/Flt-1 mRNA levels were detected in RGCs ($n = 3$) and R28 cells ($n = 8$) and (**B**) VEGFR-1/Flt-1 protein expression increased in R28 cells ($n = 5$) after 24 hours of hypoxia. (**C**) Exposure (24 hours) of cells to PEDF (500 ng/mL) and VEGF (50 ng/mL) induced VEGFR-1/Flt-1 mRNA upregulation in RGCs ($n = 3$) and R28 cells ($n = 6$), and (**D**) caused elevated VEGFR-1/Flt-1 protein levels in R28 cells ($n = 4$). (**A**, **C**) The mRNA transcripts prepared from retinal neurons were reversed transcribed and analyzed by qPCR and (**B**), (**D**) VEGFR-1/Flt-1 and β-actin expression in cell lysates of equalized cell numbers was analyzed by Western blotting. Results are expressed as relative values (fold change in VEGFR-1/Flt-1 expression; means ± SEM, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) compared with values from unstimulated (normoxic, medium) control cell cultures (*solid bars*).

PEDF Binds to VEGFR-1/Flt-1 on Retinal Neuronal Cells

Second, we determined whether PEDF interacts with VEGFR-1/Flt-1 on retinal neuronal cells. We have expressed PEDF as a protein fused to a non-Fc receptor-binding Fc fragment (hereafter referred to as PEDF-Fc; see Fig. 4A), which interacted with RGCs and R28 cells (Figs. 4B–E). Cellular binding of PEDF-Fc was largely blocked in the presence of competing PEDF and, interestingly, by a soluble VEGFR-1/Flt-1 protein/ sflt-1 (see Fig. 4B). In a colorimetric binding assay, the presence of sflt-1 in the binding buffer resulted in significantly ($P < 0.01$) reduced PEDF-Fc binding to R28 cells, compared with control conditions (see Fig. 4C), suggesting that sflt-1 and VEGFR-1/Flt-1 competed for binding to PEDF-Fc. Remarkably, preincubating the cells with both VEGF and PlGF significantly ($P < 0.05$) attenuated PEDF-Fc binding, not much less than PEDF at 10 nM ($P < 0.01$; see Fig. 4C). Moreover, we ablated VEGFR-1/Flt-1 expression in R28 cells by transfection of cells using different siRNAs. Compared with control siRNA, these siRNAs induced a 58% to 78% decrease of VEGFR-1/Flt-1 expression (see Fig. 4D). Cells displaying a knockdown of VEGFR-1/Flt-1 demonstrated a clearly reduced PEDF-Fc binding (see Fig. 4E) suggesting that VEGFR-1/Flt-1 expressed on R28 cells is involved in PEDF binding. We then immobilized sflt-1 on nitrocellulose by Western blotting and demonstrated that PEDF competed PEDF-Fc binding to sflt-1 in a binding experiment (see Fig. 5A). Furthermore, PlGF ($P < 0.01$ to $P < 0.001$) and VEGF ($P < 0.05$ to $P < 0.001$) significantly attenuated PEDF-Fc binding to immobilized sflt-1 in a solid phase binding assay (see Fig. 5B). Together, these data support the idea that PEDF is able to bind to VEGFR-1/Flt-1 and its binding site(s) overlap with those for VEGF and PlGF.

Identification of (a) PEDF Binding Site(s) in VEGFR-1/Flt-1

To identify (a) potential binding site(s) for PEDF resided in VEGFR-1/Flt-1, we generated a peptide library of soluble VEGFR-1. We identified two amino acid stretches showing binding to PEDF-Fc, which are located in the extracellular portion of VEGFR-1/Flt-1, and one stretch at the *C* terminus of soluble VEGFR-1 (Fig. 6A). These findings suggest that, in VEGFR-1/Flt-1, binding sites for PEDF-Fc do exist, being localized to a region in the third (D3) and fourth (D4) immunoglobulin-like globular domains in the extracellular portion (Fig. 6B). From the identified amino acid stretches in the extracellular part of VEGFR-1/Flt-1, we selected the peptides, NKRASVRRRIDQSNSHA (AE10) and AFITVKHRKQQVLETVA (AF10), corresponding to D3 and D4, respectively. In a subsequent competitive binding assay, we used cells of rat origin (R28), therefore, rat homologous peptides, DX21 and DW21 (see Fig. 6B), were additionally included. R28 cells were incubated with PEDF-Fc, in the presence or absence of peptides, and PEDF-Fc binding was assessed. The results shown in Figure 6C indicate that the 4 peptides were able to significantly ($P < 0.05$ to $P < 0.001$) block PEDF-Fc binding to R28 cells by 28% to 50%,

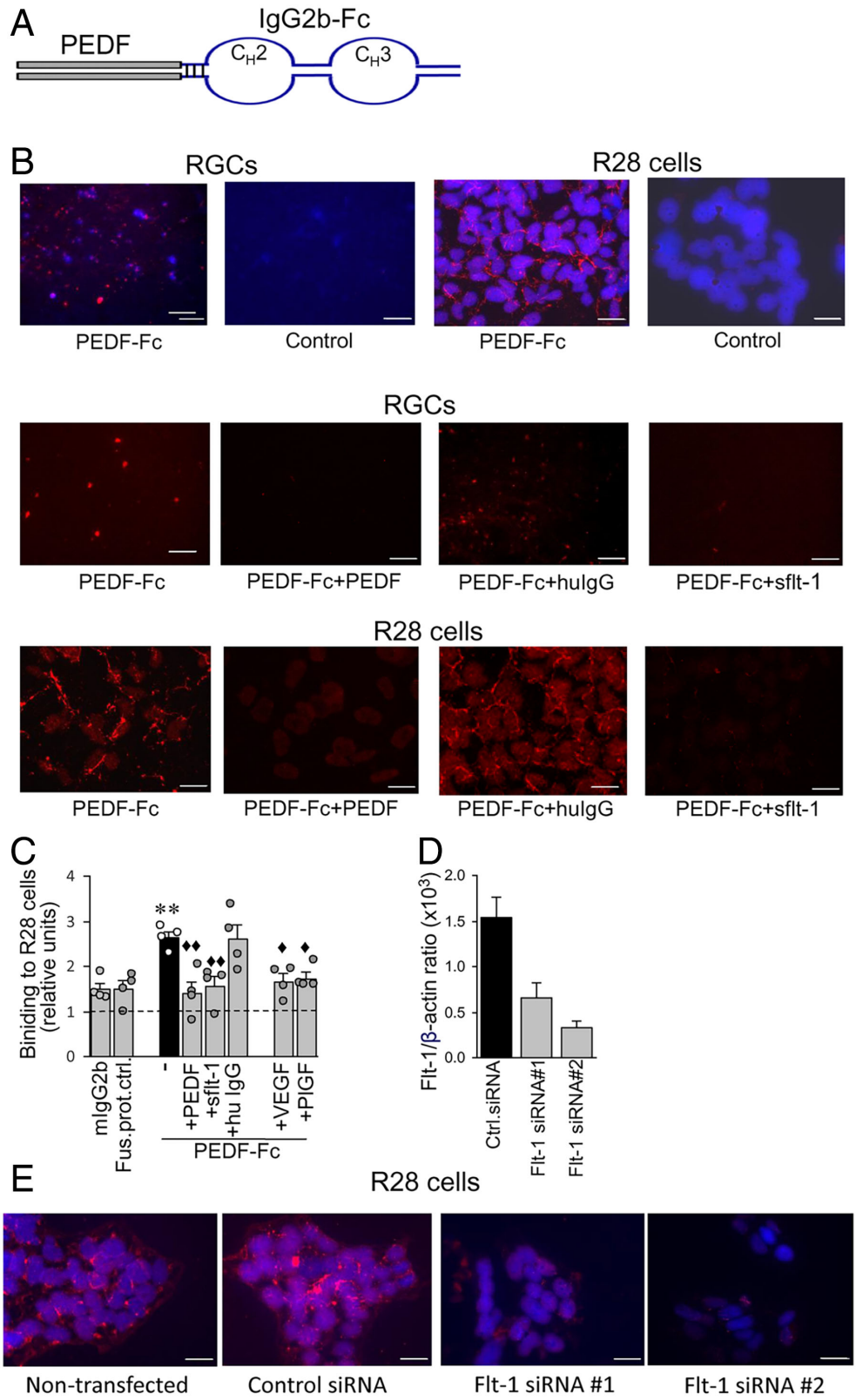

Figure 4. PEDF-Fc, a fusion protein consisting of human PEDF and hinge and constant regions of mouse (m)IgG2b heavy chain, binds to retinal neuronal cells. (**A**) Schematic representation of PEDF-Fc. (**B**) PEDF-Fc binds to RGCs and R28 cells, and binding is blocked by PEDF or sflt-1. Cells were stained with PEDF-Fc or exposed to a fusion protein control. Immunofluorescence (*red*) was detected using microscopic imaging (first line, *blue* fluorescence: cell nuclei labeled with DAPI). RGCs (second line) or R28 cells (third line) were either preincubated with 500 ng/mL PEDF or exposed to PEDF-Fc preincubated with sflt-1 or a human IgG control (each at 1 µg/mL; scale bars = 20 µm). Results are shown from one experiment of two performed with RGCs of different mice or independently cultured R28 cells. (**C**) Binding of PEDF-Fc was revealed by colorimetric immunodetection. R28 cells were preincubated either in binding buffer (–), with 500 ng/mL PEDF, 100 ng/mL

VEGF, or 50 ng/mL PlGF and subsequently exposed to PEDF-Fc. In addition, cells were left in binding buffer (*dashed line*), incubated with mIgG2b, the fusion protein control, and PEDF-Fc in the presence of sflt-1 or human IgG (control). Significant differences to values from cells exposed to mIgG2b (**$P < 0.01$) or PEDF-Fc (*black bar*; ♦$P < 0.05$, ♦♦$P < 0.01$) are indicated (means ± SEM; $n = 4$). (**D**, **E**) R28 cells were transfected with siRNAs and cultured for 48 hours. Two different VEGFR-1/Flt-1 siRNAs or control (non-transfected cells and control siRNA) conditions were applied in two independent experiments. (**D**) The effect of siRNAs on VEGFR-1/Flt-1 expression as compared to that of non-targeting control siRNA (–) was determined by qPCR (averaged values from three PCRs; error bars = standard deviation). (**E**) Knockdown of VEGFR-1/Flt-1 mRNA abolishes PEDF-Fc binding. Binding was revealed by immunofluorescence labeling and microscopic imaging (see panel **B**) and is shown for R28 cells transfected with VEGFR-1/Flt-1 siRNAs or non-transfected and control siRNA-transfected cells (scale bars = 20 µm). Cells exposed to mIgG2b or the fusion protein control demonstrated negligible reactivity (not shown).

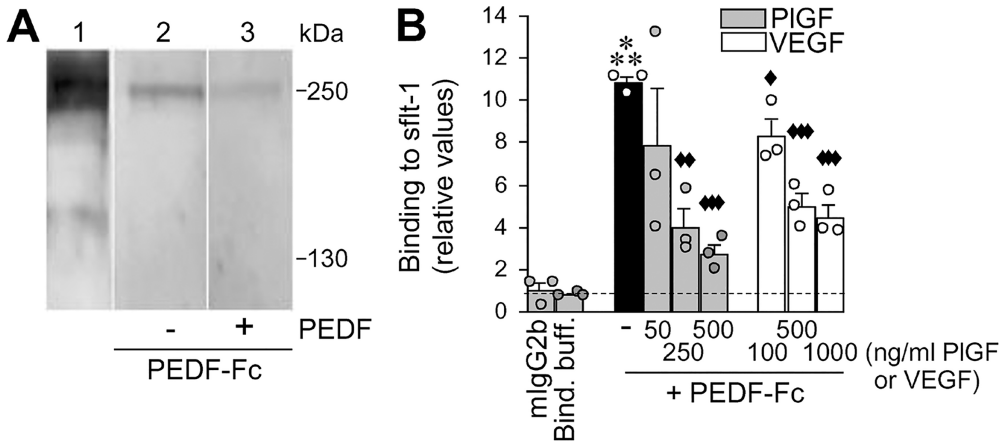

Figure 5. (**A**) PEDF competes with PEDF-Fc for binding to immobilized sflt-1. Chimeric Fc-linked human sflt-1 (400 ng per lane) was subjected to Western blotting and strips of the blot were incubated with (1) an mAb directed to human IgG or (2, 3) PEDF-Fc. A strip preincubated with PEDF prior to exposure to PEDF-Fc is shown in lane 3; sflt-1 migrated at approximately 250 kDa under non-reducing conditions. (**B**) Binding of PEDF-Fc to immobilized sflt-1 was competed by increasing amounts of PlGF or VEGF in a solid-phase binding assay. Biotinylated PEDF-Fc, in the absence (-, *black* bar) or presence of cytokines, was added to wells coated with sflt-1 and binding was revealed using streptavidin-based detection. PEDF-Fc binding is expressed as relative values (means ± SEM, $n = 3$, compared with fusion protein control, *dashed line*, ***$P < 0.001$; versus PEDF-Fc binding without cytokines, ♦$P < 0.05$, ♦♦$P < 0.01$, ♦♦♦$P < 0.001$). Control values resulting from exposure of wells to biotinylated mIgG2b control or sflt-1-replacing binding buffer are also shown.

whereas a negative control peptide had no effect. These findings strongly suggest that there are distinct regions within extracellular domains (D3 and D4) of VEGFR-1/Flt-1, which are involved in PEDF binding to VEGFR-1/Flt-1 on retinal neuronal cells.

VEGF-R1/Flt-1 is Involved in PEDF-Promoted Survival of Retinal Neuronal Cells

To evaluate whether VEGFR-1/Flt-1 is involved in PEDF-stimulated survival of retinal neurons, we investigated R28 cell survival in growth factor-deprived medium in the presence or absence of PEDF and competing sflt-1 or VEGFR-1/Flt-1-derived peptides using an MTT assay. We found that the viability of PEDF-exposed R28 cells was significantly ($P < 0.001$) reduced by approximately 20% when PEDF (10 nM) was preincubated with sflt-1. Serving as a control, cell survival stimulated by PlGF also significantly ($P < 0.05$) declined due to preincubation with sflt-1 (Fig. 7A). Additionally, we cultured R28 cells in the presence of peptides, which were presumed to act as competitors by their binding to PEDF (see Fig. 6). Peptides DX21, AE10, DW21, and AF10 at 10 µM significantly ($P < 0.01$ to $P < 0.001$) attenuated cell survival in the presence of PEDF by approximately 8% to 20% (Fig. 7B), consistent with the competing effect of sflt-1 noted before. These results suggest that PEDF can target VEGFR-1/Flt-1 and this interaction plays a significant role in mediating PEDF-promoted survival of retinal neurons.

Discussion

PEDF protects RGCs under conditions of ischemia/ reperfusion injury in vivo[12,13] and from hypoxia-induced cell death in vitro.[10,11] In the present study, we have investigated whether cytoprotective activities of PlGF and VEGF interfere with PEDF-mediated pro-survival effects. VEGF and PlGF are upregulated under hypoxic conditions in the retina.[31,33] It is therefore likely that both mediators at elevated levels co-exist with PEDF in the ischemic retina. We have found that PlGF can modulate the PEDF response in growth factor-deprived R28 cells, leading to increased ERK-1/-2 MAP kinase phosphorylation and enhanced cell survival compared to the action of either factor alone. These findings suggest that both PEDF and PlGF activate the ERK-1/-2 pathway to promote neuronal survival, making it likely that PlGF, through activation of its cognate receptor, VEGFR-1/Flt-1, may function as a signal amplifier for PEDF.

In contrast, our experiments have not shown that VEGF increases the ERK-1/-2 MAP kinase phosphorylation status in and survival of PEDF-co-stimulated retinal neuronal cells (see Figs. 1B–D) suggesting that PEDF is able to limit VEGF-promoted neuronal survival. Given that VEGFR-2/KDR-induced ERK-1/-2 signaling essentially contributes to VEGF-stimulated survival of RGCs[22,24] this finding likely reflects that PEDF prevents VEGF from binding to VEGFR2/KDR, leading to decreased VEGF-mediated effects.[40] It is also possible that pro-survival signaling pathways engaged by

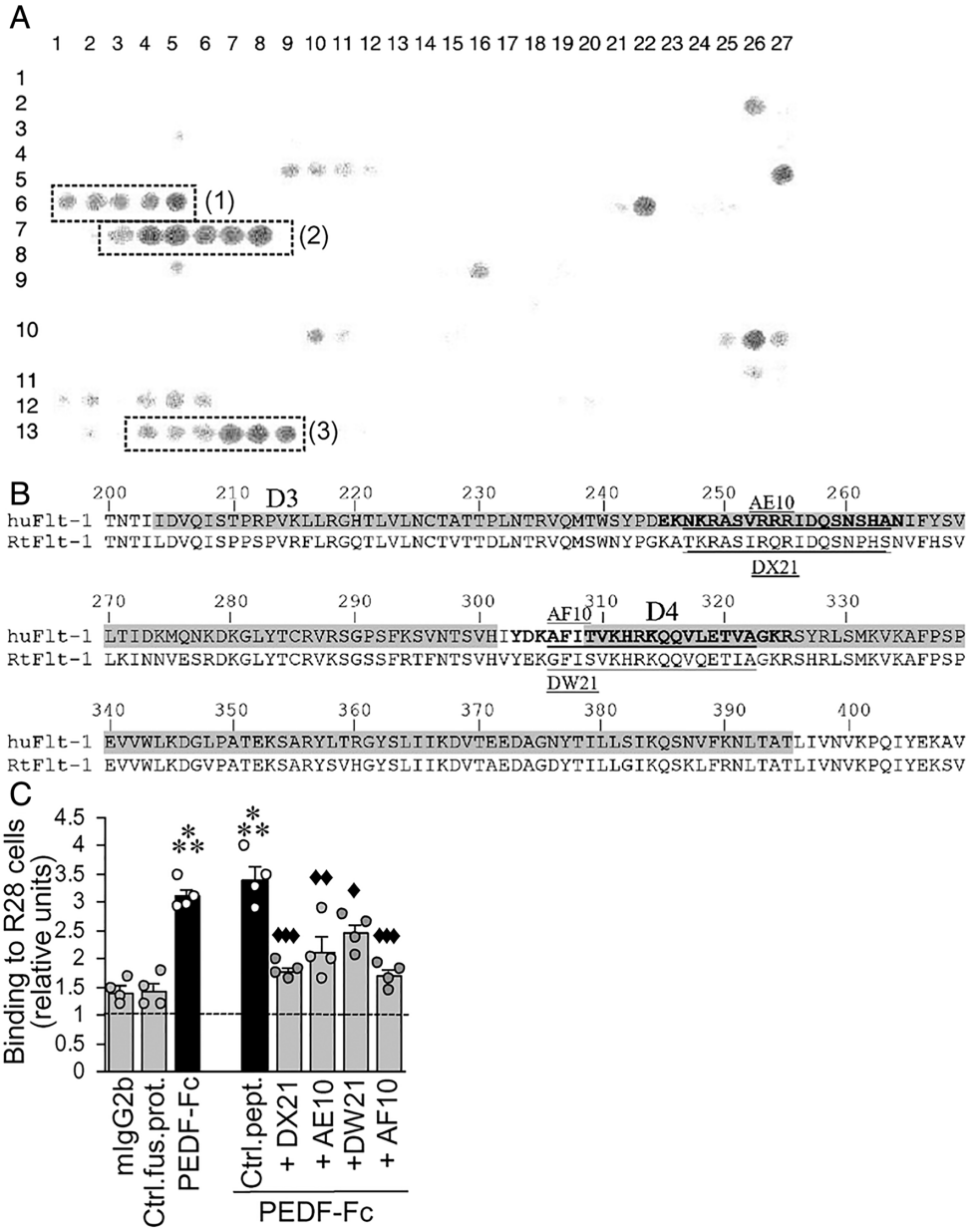

Figure 6. The amino acid sequence of human soluble VEGFR-1/Flt-1 preprotein was fragmented into 13-mer peptides, thereby shifting by two amino acids. (**A**) Interaction of PEDF-Fc with cellulose-bound peptides was revealed by chemiluminescent immunodetection. Signals in the *boxed areas* correspond to the amino acid sequences, (1) ^{245}EKNKRASVRRRIDQSNSHANI^{265}, (2) ^{303}YDKAFITVKHRKQQVLETVAGKR^{325}, and (3) ^{629}IRGEHCNKKAVFSRISKFKSTRN^{651}. (**B**) The amino acid sequence encompassing domains (*shaded in grey*) D3 (residues 204 – 301) and D4 (residues 309 – 395) of mature human VEGFR-1/Flt-1 (huFlt-1) was aligned with the homologous sequence of mature rat VEGFR-1/Flt-1 (RtFlt-1). Sequences used for alignment were obtained from Genbank (accession numbers NP_002010.2 and NP_062179.2). Amino acid numbering of human VEGFR-1/Flt-1 is related to Ser as the first amino acid of the mature protein. Sequence stretches (1) and (2) that interact with PEDF-Fc (see panel A) are indicated in *bold*. *Underlined* amino acids constitute peptides AE10, AF10, DX21, and DW21 used in a competitive binding assay as shown in panel (**C**): the peptides (each at 10 µM, based on prior findings in RPE cells) competed binding of PEDF-Fc to R28 cells. PEDF-Fc was preincubated with AE10, AF10, DX21, and DW21 or a control peptide (*solid bar*) and further incubated with R28 cells overnight. As controls, cells were either left in binding buffer (*dashed line*) or incubated with mIgG2b, a control fusion protein or PEDF-Fc. Binding of PEDF-Fc was revealed by colorimetric immunodetection. Significant differences to cells exposed to mIgG2b (***$P < 0.001$) or PEDF-Fc in the presence of a control peptide ($^{\blacklozenge}P < 0.05$, $^{\blacklozenge\blacklozenge}P < 0.01$, $^{\blacklozenge\blacklozenge\blacklozenge}P < 0.001$) are indicated (means ± SEM; $n = 4$).

both PEDF and VEGF are operative but interfere with each other, thereby limiting cooperative signaling of both factors in stimulating cell survival. In retinal endothelial cells, however, PEDF is able to reduce VEGFR-1/Flt-1 autophosphorylation, in the presence of VEGF.[41] If this is a relevant PEDF activity in neuronal cells, it may also explain suppression of VEGF-induced downstream (ERK-1/-2) signaling.

After combined treatment with PEDF and VEGF, cell survival changed to a different degree in RGCs compared to R28 cells (see Figs. 1B, 1C). PEDF- and VEGF-stimulated

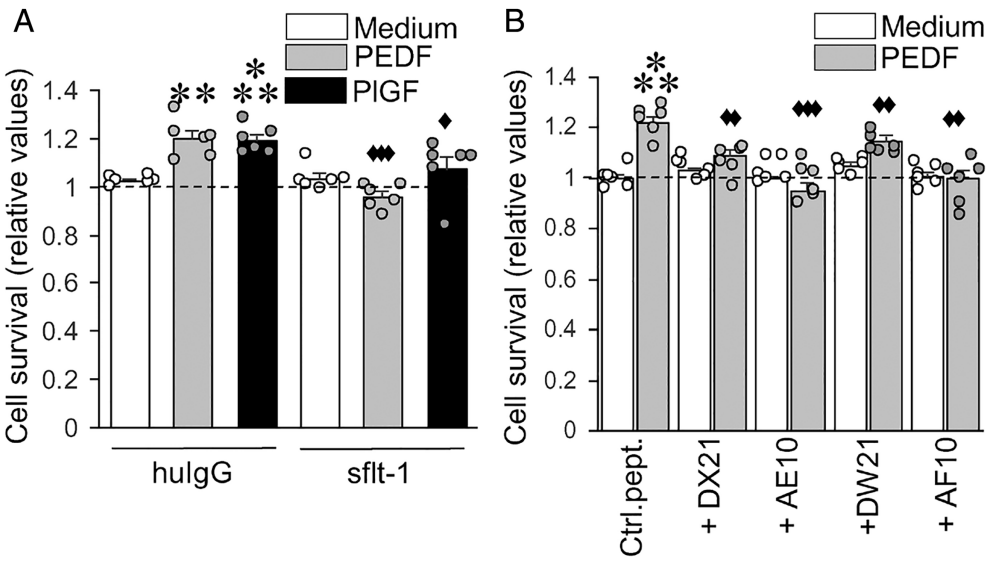

Figure 7. VEGFR-1/Flt-1 is involved in PEDF-promoted survival of R28 cells. PEDF (500 ng/mL) was preincubated with (**A**) sflt-1 (see legend to Fig. 5A) or (**B**) VEGFR-1/Flt-1-derived peptides. Samples were then incubated with growth factor-deprived R28 cells for 24 hours and cell survival was determined by MTT assays. Results are expressed as relative values in relationship to values from medium control cultures (*dashed lines*). Data are representative of six experiments using independent R28 cell cultures. (**A**) Sflt-1 (50 ng/mL) attenuates enhanced cell survival induced by PEDF or PlGF (50 ng/mL). Significant differences to control cultures (presence of human IgG, huIgG, 50 ng/mL; $^{\blacklozenge}P < 0.05$, $^{\blacklozenge\blacklozenge\blacklozenge}P < 0.001$) and the effects of PEDF and PlGF in huIgG control cultures ($^{**}P < 0.01$, $^{***}P < 0.001$) are indicated (means ± SEM; $n = 6$). (**B**) VEGFR-1/Flt-1 peptides (10 µM) inhibit R28 cell survival promoted by PEDF. The effect of DX21, AE10, DW21, and AF10 compared to that of a control peptide ($^{\blacklozenge\blacklozenge}P < 0.01$, $^{\blacklozenge\blacklozenge\blacklozenge}P < 0.001$) and the effect of PEDF in control cultures ($^{***}P < 0.001$) are shown (means ± SEM; $n = 6$).

signaling may be differently modulated by endogenous factors in RGCs and R28 cells; these factors may include PEDF[47] and VEGF[48] themselves at different relative levels. Effects related to species differences of the cells (mouse or rat) and their distinct interaction with both survival factors (human PEDF and rat VEGF) can also not be excluded at present.

PlGF has been shown to exert anti-apoptotic effects on retinal neurons and RPE cells through VEGFR-1/Flt-1 in vitro.[25,26] We therefore assumed that this receptor is involved in PlGF-modulatable pro-survival activities of PEDF (see above). Our experiments strongly suggest that PEDF can bind to VEGFR-1/Flt-1 expressed by RGCs or R28 cells (see Figs. 4B, 4C, 4E, 6C). Identifying amino acid sequences in the extracellular portion of VEGFR-1/Flt-1 interacting with PEDF-Fc allows to conclude that PEDF binding sites are located in domains D3 and D4. VEGFR-1/Flt-1 possesses seven extracellular immunoglobulin homology domains (D1–D7). Domains D2 (ligand binding mediating domain) and D3 are known to be required for efficient receptor interactions with VEGF and PlGF.[49–51] Thus, PEDF binding to VEGFR-1/Flt-1 seems to require accessibility of a distinct region (D4) and overlaps, at least partially, the region involved in VEGF and PlGF binding. Although more investigation is necessary to explore the signaling pathway(s) by which VEGFR-1/Flt-1 is involved in PEDF-promoted cell survival of retinal neurons our study provides first evidence that, through binding of VEGFR-1/Flt-1, PEDF can exert neuroprotective effects. However, VEGFR-1/Flt-1 is co-expressed with further PEDF-binding molecules, that is, adipose triglyceride lipase/ATGL[52] and 37/67-kDa laminin receptor[53] in retinal neuronal cells including RGCs.[47,53,54] It is possible that all these receptors may serve distinct functions in regulating PEDF-mediating signaling and neuronal survival.

It is conceivable that future PEDF-based therapeutic neuroprotection will take advantage of a synergistic interplay with (an)other neuroprotective factor(s). PlGF is a candidate molecule for a synergistically acting neuroprotectant and should be taken into account in therapeutic regimens. Considering a role for its receptor, VEGFR-1/Flt-1, in ensuring neuronal cell integrity, it will be essential to maintain in therapy approaches its inherent tyrosine kinase (and signaling) activity. However, currently used therapeutic VEGF neutralization in the treatment of neovascular age-related macular degeneration (AMD) and macular edema includes recombinant PlGF-binding fusion proteins (aflibercept/VEGFtrap and conbercept), which contain VEGF-/PlGF-interacting domain D2 of VEGFR-1/Flt-1 and act as soluble decoy receptors. PlGF plays a pro-angiogenic role in neovascular AMD and in the ischemic retina.[55,56] Because VEGF and PlGF recruit different downstream signaling pathways an "anti-angiogenic targeting" of PlGF may be preferable, but further studies should aim to examine whether a long-term blockade of PlGF-VEGFR-1/Flt-1 interaction may lead to unwanted side effects on the neuroretina.

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