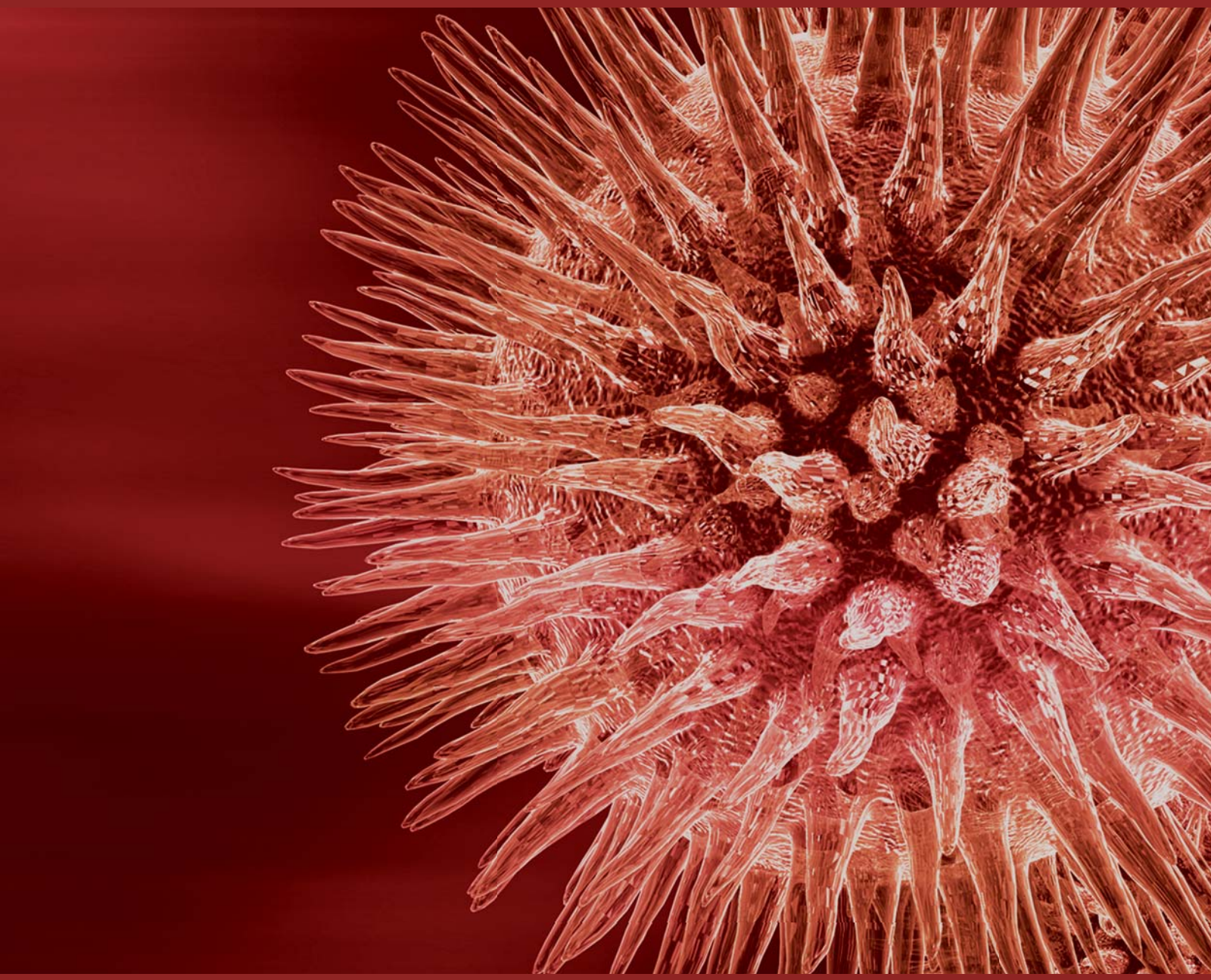
Journal of Biomedicine and Biotechnology

Pigment Epithelium-Derived Factor: Chemistry, Structure, Biology, and Applications

Guest Editors: S. Patricia Becerra, Crispin R. Dass, Takeshi Yabe, and Susan E. Crawford



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Contents

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Editorial

Pigment Epithelium-Derived Factor: Chemistry, Structure, Biology, and Applications

S. Patricia Becerra,[1] Crispin R. Dass,[2] Takeshi Yabe,[3] and Susan E. Crawford[4]

[1] *Section of Protein Structure and Function, Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, Bethesda, MD 20892, USA*
[2] *School of Biomedical Sciences, Victoria University, Melbourne, VIC, Australia*
[3] *Department of Medical Pharmacy, Setsunan University, Osaka 573-0101, Japan*
[4] *Department of Pathology, St. Louis University, St. Louis, MO 63104, USA*

Correspondence should be addressed to S. Patricia Becerra, becerrap@nei.nih.gov

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Pigment epithelium-derived factor (PEDF) is a multifunctional serpin protein with demonstrable neurotrophic, antiangiogenic, antitumorigenic, antimetastatic, anti-inflammatory, antioxidative properties among others. PEDF exists naturally in most organs of the human body, and it is released from most cell types as an extracellular diffusible and circulating glycoprotein. It has been studied mostly in the eye, where its levels are altered in diseases characterized by retinopathies, such as age-related macular degeneration and diabetic retinopathy. The importance of PEDF in the development, maintenance, and function of the retina and CNS is evident in animal models for inherited and light-induced retinal degeneration, as well as for degeneration of spinal cord motor neurons. Pathological ocular neovascularization- and retinal degeneration-related animal models have prompted clinical development. Clinical trials to assess the safety of a viral expression vector for PEDF in the context of age-related macular degeneration have been performed. Interestingly, PEDF has multiple biological effects against tumors, and its efficacy has been demonstrated in several animal models for tumor growth and progression. The mechanisms of PEDF action on tumors have been associated to inhibition of tumor angiogenesis, and also negative effects directed on tumor cells. Recently, the involvement of PEDF in stem cell biology has been revealed. Moreover, PEDF is a potential diagnostic tool for several diseases triggered by pathological neovascularization, retinal degenerations, or tumors.

The present issue includes three original research and four review articles. In one article, V. Chandolu and C. R. Dass review the biological functions of PEDF against cancer, with a focus on a particular type of bone cancer called osteosarcoma. They summarize the progress in understanding the function of PEDF in antiangiogenesis, tumor cell differentiation, and direct tumor cell inhibition. In another review article, J.-T. Liu et al. address the role of PEDF in stem/progenitor cell-associated neovascularization, in particular in cardiovascular and neurovascular biology. M. Elahy et al. review the promising significance of PEDF in stem cell biology, specifically in human embryonic stem cells, mesenchymal stem cells (MSCs), neural stem sells (NSCs), and stem cells overexpressing the PEDF gene. X.-F. Zhu et al. summarize the advances of PEDF in diabetic retinopathy, its protective effect on oxidative stress, which is the main trigger for the pathology, and the potential application of PEDF in diabetic retinopathy. In a research article, P. Subramanian et al. report the identification of biochemical forms of PEDF with distinct biological effects on tumor cells, which may explain the multifunctional modality of this protein. In a second research article, M. L. Broadhead et al. report the effects of two PEDF-derived peptides on a clinically relevant murine model of osteosarcoma with spontaneous metastasis. Finally, T. Falk et al. report that human retinal pigment epithelial cells grown on plastic-based microcarriers retain the ability to produce both PEDF and vascular endothelial growth factor VEGF-A. This constitutes a novel candidate

delivery system for neurotrophic factors with potential application in neurodegenerative diseases, such as Parkinson's.

In summary, given the multimodal nature of PEDF, the present issue aims to enhance our understanding of the chemistry, structure, biology, and application of PEDF in biomedicine. It gives an overview of the current status of research on PEDF and will prove useful as source of reference for students and researchers.

S. Patricia Becerra
Crispin R. Dass
Takeshi Yabe
Susan E. Crawford

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Research Article

Identification of Pigment Epithelium-Derived Factor Protein Forms with Distinct Activities on Tumor Cell Lines

P. Subramanian,[1] M. Deshpande,[1] S. Locatelli-Hoops,[1] S. Moghaddam-Taaheri,[1] D. Gutierrez,[2] D. P. Fitzgerald,[3,4] S. Guerrier,[1] M. Rapp,[1] V. Notario,[5] and S. P. Becerra[1]

[1] *Section of Protein Structure and Function, LRCMB, National Eye Institute, NEI, Bethesda, MD 20892-0608, USA*
[2] *Laboratory of Retinal Cell and Molecular Biology, National Eye Institute, NEI, Bethesda, MD 20892, USA*
[3] *Center for Cancer Research (CCR), National Cancer Institute, Bethesda, MD 20892, USA*
[4] *Otsuka Maryland Medicinal Laboratories, Rockville, MD 20850, USA*
[5] *Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC 20057, USA*

Correspondence should be addressed to S. P. Becerra, becerrap@nei.nih.gov

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Purpose. Pigment epithelium-derived factor (PEDF) is a multifunctional serpin. The purpose of this study is to identify PEDF protein forms and investigate their biological activities on tumor cell lines. *Methods.* Recombinant human PEDF proteins were purified by cation- and anion-exchange column chromatography. They were subjected to SDS-PAGE, IEF, deglycosylation, heparin affinity chromatography, and limited proteolysis. Cell viability, real-time electrical impedance of cells, and wound healing assays were performed using bladder and breast cancer cell lines, rat retinal R28, and human ARPE-19 cells. *Results.* Two PEDF protein peaks were identified after anion-exchange column chromatography: PEDF-1 eluting with lower ionic strength than PEDF-2. PEDF-1 had higher pI value and lower apparent molecular weight than PEDF-2. Both PEDF forms were glycosylated, bound to heparin, and had identical patterns by limited proteolysis. However, PEDF-2 emerged as being highly potent in lowering cell viability in all tumor cell lines tested, and in inhibiting tumor and ARPE-19 cell migration. In contrast, PEDF-1 minimally affected tumor cell viability and cell migration but protected R28 cells against death caused by serum starvation. *Conclusion.* Two distinct biochemical forms of PEDF varying in overall charge have distinct biological effects on tumor cell viability and migration. The existence of PEDF forms may explain the multifunctional modality of PEDF.

1. Introduction

Pigment epithelium-derived factor (PEDF) is a 50 kDa secreted glycoprotein and a member of the serpin superfamily with no demonstrable protease inhibitory activity [1]. PEDF is associated with several biological processes due to its antiangiogenic, anti-inflammatory, anti-oxidative, neurotrophic, and neuroprotective properties [2]. Moreover, it has been implicated in another interesting role, as an antitumor and antimetastatic agent with applications in multiple malignancies such as retinoblastoma, lung, breast, prostate, ovarian and pancreatic carcinomas, uveal melanoma, glioma, and osteosarcoma [3–8]. PEDF levels are decreased in tumor cells relative to normal cells, and PEDF addition inhibits tumor formation and metastasis, blocks angiogenesis, and induces apoptosis in tumor and endothelial cells. In contrast, PEDF promotes retinal cell survival and neuronal differentiation, protects retinal pigment epithelial cells against oxidative stress [2, 3, 9] and plays a role in expansion of neural stem cells [10]. The mechanisms that mediate the multimodal functionality to PEDF are not clear.

Previous structure-function studies revealed that two peptide regions toward the amino end of the PEDF polypeptide have distinct biological activities [2, 11, 12]. The peptide region termed 34-mer (amino acid residues Asp^{44}-Asn^{77} of the human PEDF sequence) forms alpha helix A of the 3D structure of human PEDF [13] and confers antiangiogenic and antitumorigenic properties to the PEDF polypeptide

[12]. The 44-mer peptide (Val^{78}-Thr^{121}) derived from the region that forms alpha helices B, C, and part of D exhibits neurite-outgrowth activity and protects spinal cord motor neurons against chronic toxicity [11, 14]. A smaller peptide derived from amino acid positions 82–121 exhibits effective neuroprotective properties in retinal ischemia [15].

Interestingly, the native PEDF has several isoforms differing in isoelectric point (pI) values [16, 17] implying differences in posttranslational modifications of the polypeptide backbone. Duh et al. reported that the secreted human recombinant PEDF from human embryonic kidney (HEK) cells has at least two species varying in their carbohydrate composition of the N-glycosylation site and efficacy of suppressing vascular endothelial growth factor-induced proliferation and migration of retinal microvascular endothelial cells [18]. Maik-Rachline and Seger showed that the human plasma PEDF is a phosphoprotein and that extracellular phosphorylation converts the recombinant protein from a neurotrophic to an antiangiogenic factor [19]. Konson et al. demonstrated that a triple phosphomimetic-altered PEDF is more efficient than wild-type PEDF in inhibiting neovascularization and tumor growth *in vivo* and suppresses cultured endothelial cell proliferation and cell migration much better than the wild-type PEDF [20].

Given the above, the aim of this study was to identify and characterize PEDF isoforms that could contribute to the complexity of PEDF action and its multifunctional modality.

2. Methods

2.1. Cell Culture. Human bladder carcinoma T24 cells (Cell Line collection, Lombardi Comprehensive Cancer Center, passage 33), human breast cancer 231-BR cells [21] (passage 13), mouse breast cancer 4T1-BR5 cells [21] (passage 11), human breast tumor MDA-MB-231 cells (Cell Line Collection, Lombardi Comprehensive Cancer Center, passage 6), mouse breast cancer 4T1 cells (Cell Line Collection, Lombardi Comprehensive Cancer Center, passage 9), and rat retinal R28 (generous gift of Gail Seigel, passages 47–55), were cultured in DMEM medium. Human retinal pigment epithelial ARPE-19 cells (American Type Culture Collection, passages 27–32) were cultured in DMEM-12 medium. Media were supplemented with 10% of fetal bovine serum (FBS) and 1% penicillin/streptomycin and cultures were incubated at 37°C with 5% CO_2.

2.2. Protein Purification. Recombinant human PEDF was purified from the culture media of BHK cells harboring an expression plasmid containing full-length PEDF cDNA [22]. The culture media was concentrated by ammonium sulfate precipitation and subjected sequentially to cation- and anion-exchange column chromatography as described before with the following modifications [13]. Cation-exchange column chromatography was performed using a POROS S resin connected to a BioCAD 700E perfusion chromatography system, with buffer S (20 mM Na phosphate, pH 6.5, 50 mM NaCl) and elutions were with a linear gradient of 50–500 mM NaCl in Buffer S. PEDF-containing fractions were pooled, dialyzed against buffer Q (50 mM Tris-HCl, pH 8) and subjected to POROS Q column chromatography in buffer Q and elutions were done with a linear gradient of 100–300 mM NaCl in buffer Q. The PEDF-containing fractions were pooled, concentrated, and the buffer exchanged to PBS using ultrafiltration devices (Centricon-30 or Amicon-30, Millipore). Storage of the final samples was at −80°C.

2.3. Protein Analyses. Proteins were analyzed by SDS-PAGE using 10–20% polyacrylamide in SDS-Tricine gels (Invitrogen) or NuPAGE 10% polyacrylamide gel in Bis-Tris buffer with NuPAGE MOPS-SDS as running buffer (Invitrogen) under reducing conditions, and isoelectric focusing (IEF). Protein detection was accomplished with Coomassie Blue stain. PEDF protein identity was confirmed by Western Blot. After separation by SDS-PAGE electrophoresis, proteins were transferred to a nitrocellulose membrane, blocked for 1 h at room temperature, and incubated with polyclonal antibody to PEDF (Bioproducts MD, Inc.) in blocking solution at 1 : 5,000 or 1 : 10,000 dilution, followed by secondary antibody anti-rabbit IgG (H + L) in a 1 : 1000 dilution and the Vectastatin ABC Kit (Vector Laboratories) with colorimetric detection reagent 4-chloro-1-naphthol (BioRad Laboratories) as substrate. Alternatively, the secondary antibody was affinity-purified peroxidase-labeled goat anti-rabbit IgG (H + L) in a 1 : 200,000 dilution, and chemiluminescence detection with Super Signal West Dura Extended Duration Substrate (Thermo Scientific) on X-ray films.

Protein concentrations were determined using the Protein Assay Kit (BioRad) and Beckman DU 640 Spectrophotometer. Protein concentration was calculated from absorbance values using the formula:

$$\begin{aligned}&[\text{Absorbance}_{595\,\text{nm}} \times 3.5\,\mu\text{g BSA}]\\ &\quad \div [\text{Sample volume } (\mu\text{L}) \times 0.2]\\ &\quad = \text{protein concentration (mg/mL)}.\end{aligned} \tag{1}$$

Isoelectric focusing was performed using pH 3–7 or pH 3–10 IEF gels (Invitrogen), following manufacturer's instructions.

2.4. Enzymatic Deglycosylation. Two micrograms of protein was treated with N-glycosidase F (New England Biolabs, Ipswich, MA) following a previously described method [17]. Briefly, the protein sample was denatured by boiling in a solution containing 0.5% SDS, 40 mM DTT. To avoid inactivation of the enzyme by SDS, a total of 1% NP-40 in 50 mM sodium phosphate, pH 7.5 was added to the denatured sample before adding 1000 Units of N-glycosidase F in 20 μL final reaction volume. Enzymatic reactions were incubated at 37°C for 1 h followed by addition of SDS-PAGE sample buffer and samples were boiled for 10 min.

2.5. Limited Proteolysis. PEDF was cleaved with limiting amounts of chymotrypsin. Reaction mixtures contained chymotrypsin (Worthington, Lakewood, NJ) and PEDF substrate: at a w/w ratio of 1 : 10 in 80 mM Tris-HCl, pH 7.5 and

100 mM $CaCl_2$. Protein amounts were 1 μg and incubation temperature was 25°C. Incubation times were as indicated. The reactions were stopped by freezing in dry ice and the addition of SDS-PAGE sample buffer. Reaction products were analyzed by SDS-PAGE analysis of products.

2.6. Glycosaminoglycan-Binding Assays. Heparin-binding assays were performed using heparin affinity column chromatography as described previously [23]. Hyaluronan-binding assays were performed using hyaluronan affinity column chromatography as described before [24].

2.7. RT-CES Assay. The cells were plated on a microplate biosensor platform and real-time electrical impedance of cells was followed with RT-CES system from ACEA Biosciences (San Diego, CA) (http://www.aceabio.com), a system described previously [25, 26]. T24, 231-BR, and 4T1-BR5 cells were seeded at 6,000 cells per well (a 96-well format) and allowed to attach for 18 h. Then the cells were serum-starved for 8 h followed by addition of serum-free media containing PEDF. R28 cells were seeded at a density of 3000 cells/well on 16-well strips of 96-well format with microelectrodes. Background impedance was measured with media alone before adding the cells. The cells were allowed to attach for 8 h with media containing 5% FBS. This was followed by changing to media with or without PEDF at the desired concentration. Real-time electrical cell impendence was monitored every hour in each well for several hours. Data from two replicates was averaged.

2.8. Cell Viability Assays. At the end point of the RT-CES assay, cell viability was measured by determining the relative levels of intracellular ATP as a biomarker for live cells using a CellTiter-Glo kit (Promega, Madison, WI) and following instructions by manufacturer. After 30 min of incubation at room temperature, the incubation solution in each well was transferred into wells in a 96-well microtiter plate. The luminescence intensity was measured using an automated plate reader (Envision, Perkin Elmer, MA). In parallel experiments, cells cultured in 24-well plates in triplicates were imaged and at the end point, cell viability was measured by determining the relative levels of mitochondrial dehydrogenase activity as live cell biomarker using the Cell Counting Kit-8 (Dojindo) following instructions by manufacturer. Cells in each well were incubated with 50 μL of CCK-8 solution diluted 1 : 25 and incubated for 4 h at 37°C. Absorbance of each well was measured at 450 nm using an automated plate reader (Envision). In all the cases, the absorbance reading for background was subtracted from the readings of samples. Data from replicates were averaged and statistical analysis was performed by a t-test. A P value of <0.05 was taken as significant.

2.9. In Vitro Migration Assay. Cell migration was assessed using wound-healing assays. Confluent cultures of ARPE-19 cells on 24-well culture plates and of MDA-MB-231 cells in 6-well plates were scratched with 10-μL pipette tips to create fixed-width linear "wounds" in the cell monolayers, followed by incubation with medium containing indicated concentrations of PEDF. Cell migration was monitored by capturing the images of linear wound closure using a 2X brightfield objective in an Olympus 1 × 70 inverted microscope coupled with a Sony camera. Images were taken immediately after wounding and later at defined time intervals until complete closure in the control cultures.

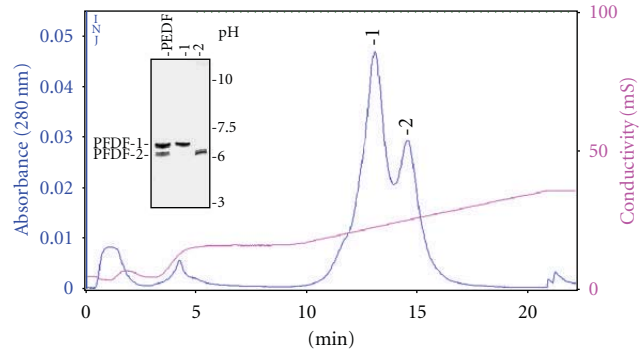

Figure 1: Anion-exchange Column Chromatography of PEDF. Chromatogram of PEDF with a POROS HQ column attached to BioCad700, running buffer of 50 mM Tris-HCl, pH 8, and a flow rate of 3 mL/min. Elution was with 25 column volumes of a linear gradient from 100 to 300 mM NaCl. On the chromatogram, blue indicates the O.D. of the sample read at 280 nm, magenta indicates the conductivity. Resolution of load and peak fractions by IEF is shown in the inset.

2.10. Mass Spectrometry. Matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry was performed to determine the average molecular mass of the two PEDF protein forms. Purified samples of PEDF-1 (0.8 μg/μL) and PEDF-2 (2.5 or 1 μg/μL) in PBS were mixed 1 : 3 (v/v) with a saturated solution of sinapinic acid in 50% acetonitrile, 0.3% trifluoroacetic acid, and 0.5 μL of each sample was spotted onto a stainless steel MALDI plate. Mass spectra were manually acquired with 100 laser shots/spectrum using a MALDI Voyager DE-STR (AB Sciex) in positive ion, linear mode. The spectra were calibrated using a mixture of BSA and IgG calibration standards (AB Sciex). Additionally, some samples were mixed 1 : 1 (v/v) with the standard and calibrated internally, postacquisition. Molecular masses of both forms of PEDF were determined by averaging the means of 5 spectra collected from each of three sample spots.

3. Results

3.1. Identification of Two Forms of the PEDF Protein. Human recombinant PEDF was purified from culture media by a two-step procedure involving sequential cation- and anion-exchange column chromatography. The chromatogram from the cation-exchange column showed one broad peak of PEDF eluting with 200–250 mM NaCl, which was pooled and prepared for anion-exchange column chromatography. The anion-exchange column chromatogram showed two main protein peaks (Figure 1). Using conductivity values for the

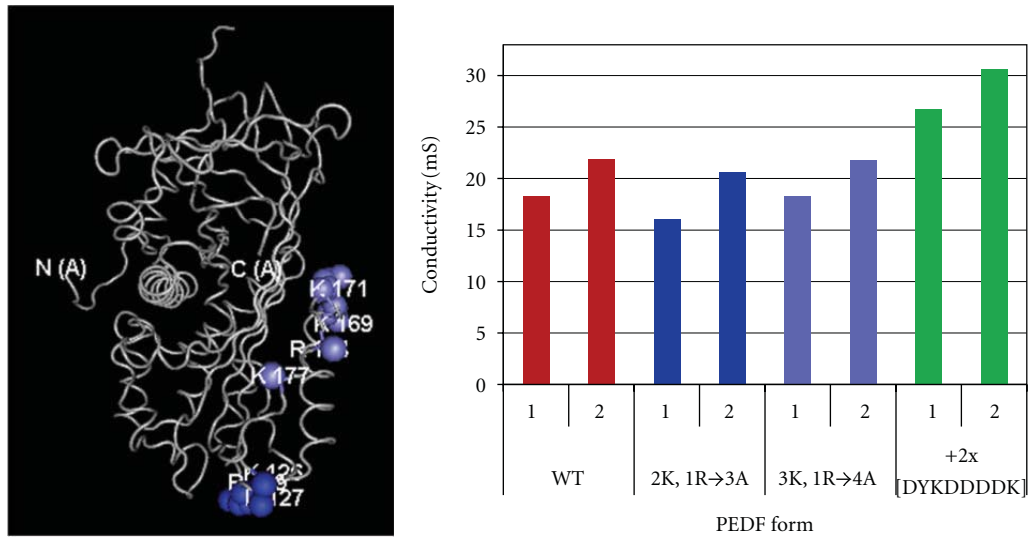

Figure 2: Recombinant altered PEDF proteins secreted to the culturing media were analyzed by anion-exchange column chromatography. Altered PEDF proteins were K146/K147/R149 (2 K,1R→3A) and K189/K191/R194/K197 (3 K,1R→4A), and FLAG-PEDF. (a) the backbone of the 3D structure of PEDF (tube in grey) from PDB 1IMV with highlighted location for amino acids K146/K147/R149 (blue) and K189/K191/R194/K197 (light blue). FLAG tagged PEDF had two tandem repeats of DYKDDDDK on the carboxy-end (C(A)). (b) Conductivity of PEDF fractions at peaks by anion-exchange column chromatography was determined and plotted.

fractions (18.3 mS and 21.96 mS) we determined that protein in the first and second peaks eluted with about 200 mM NaCl and 253 mM NaCl, respectively. Isoelectric focusing of the proteins revealed a higher pI value for the protein in the first peak (peak 1) (pI = 7) than in the second one (peak 2) (pI = 6.1). Proteins in fractions from the two peaks contained PEDF-immunoreactive bands migrating as ~50,000-Mr by SDS-PAGE (see Figure 3). In addition to the IEF gel, Coomassie blue stained SDS-PAGE gels demonstrated that the PEDF forms were highly pure, and the proteins from the first peak migrated slightly faster than those in the second peak (see Figure 3). The protein eluting with lower ionic strength was termed PEDF-1, and the version eluting with higher ionic strength was labeled PEDF-2. As determined by MALDI-TOF mass spectrometry, the average molecular mass (mean ± standard error) of PEDF-1 was 46,063 ± 13 Da and the molecular mass of PEDF-2 was 47,176 ± 87 Da, in agreement with the differences in migration by SDS-PAGE.

To find out how surface exposed charges affect fractionation of PEDF, alterations at positively charged residues K146/K147/R149 and K189/K191/R194/K197 of PEDF to neutral side chain residue alanine were prepared. Altered proteins were as described before [24]. Fractionation by anion-exchange column chromatography showed that in both cases two peaks of PEDF were eluted with conductivity values similar to those of wild-type PEDF (Figure 2). Recombinant PEDF fused with two tandem repeats of FLAG peptide at the carboxy-end produced by HEK cells [21] was also subjected to anion-exchange column chromatography under identical conditions as above. FLAG peptide (DYKDDDDK) with several acidic amino acid residues would increase the overall negative charge of the PEDF protein. We found that FLAG-PEDF eluted in two peaks with higher ionic strength than the untagged PEDF (with 316 mM NaCl and 367 mM NaCl, resp.) (Figure 2). These results indicate that recombinant human PEDF contains at least two isoforms varying in charge and in apparent molecular weight, and that this characteristic is general.

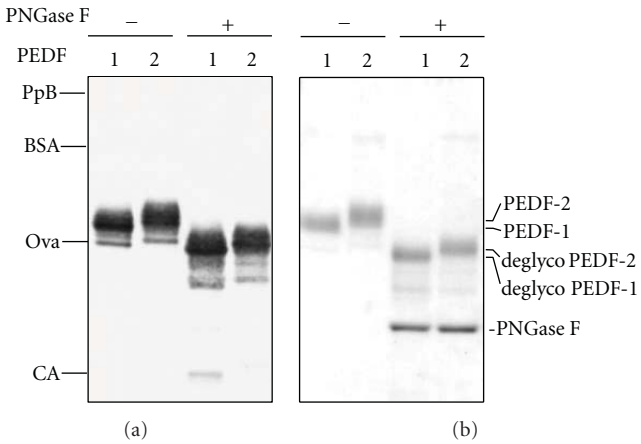

Figure 3: Deglycosylation of PEDF-1 and PEDF-2: deglycosylation of PEDF proteins was performed with PNGase F under denaturing conditions. Proteins were resolved by SDS-PAGE. (a) Western blotting of untreated and treated PEDF proteins (0.15 μg per lane) with anti-PEDF. (b) Coomassie blue staining of untreated and treated PEDF proteins (1.5 μg per lane). Migration position of PNGaseF is indicated on the right side, and molecular weight markers are on the left.

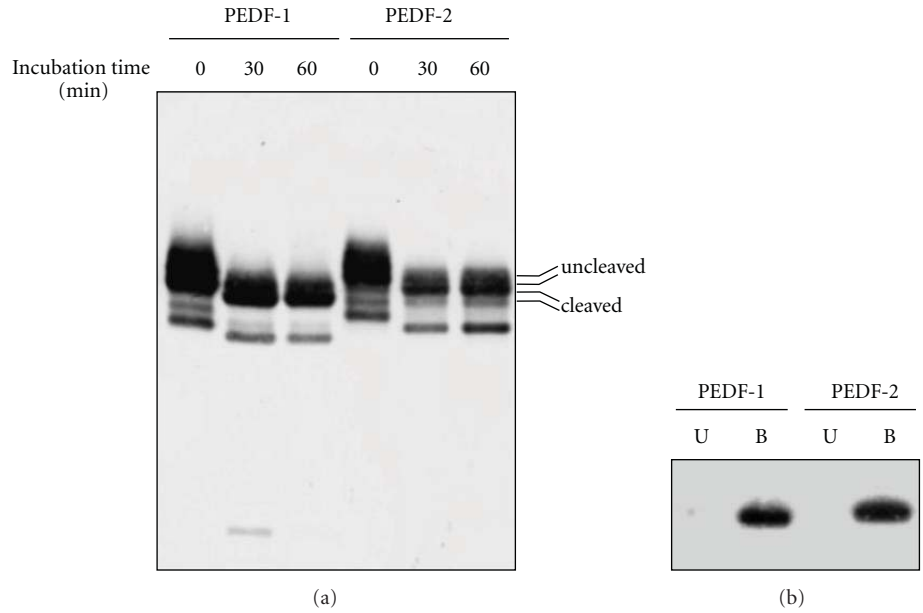

Figure 4: (a) Chymotrypsin Limited Proteolysis: reactions for the indicated times were performed with mixtures containing α-chymotrypsin and PEDF substrate at a ratio of 1 : 10 (w/w). Reactions were resolved by SDS-PAGE (0.1 μg per lane) followed by western blotting with anti-PEDF. Cleaved and the uncleaved forms are shown to the right. (b) Binding to Heparin. PEDF-1 and PEDF-2 were subjected to heparin-affinity column chromatography. Unbound (U) and bound (B) material was analyzed by SDS-PAGE and immunostained with anti-PEDF.

3.2. Deglycosylation of PEDF-1 and PEDF-2. To determine whether the glycosylation attachments on the PEDF-1 and PEDF-2 polypeptide backbones are involved in their differences, the proteins were treated with N-glycosidase F to release Asn-linked oligosaccharides from glycoproteins. Figure 3 shows that N-glycosidase F digested both PEDF-1 and PEDF-2 causing them to migrate faster by SDS-PAGE. It indicates that the enzymatic treatment decreased their apparent molecular weight by identical values of ~8000. Both PEDF forms were glycosylated, and the deglycosylated PEDF-1 still migrated slightly faster than PEDF-2 by SDS-PAGE. These observations demonstrate that each PEDF-1 and PEDF-2 molecule had similar carbohydrate content of about 15% attached to an internal asparagine residue and that it does not account for the differences between the two forms PEDF-1 and PEDF-2.

3.3. Limited Proteolysis and Heparin Binding. The overall protein confirmation of both PEDF forms was analyzed by limited proteolysis. Treatment with limiting amounts of chymotrypsin revealed that both PEDF forms had identical patterns after digestion. By incubation with chymotrypsin at a protease : substrate ratio of 1 : 10 (w/w), both PEDF forms of about 50 kDa were digested in a one-step fashion into a product of about 46 kDa, that is, a decrease of ~4 kDa. Complete substrate utilization was achieved by 60 min (Figure 4(a)). A band corresponding to the released low-molecular-weight peptide was not readily identified by Coomassie Blue staining of the gel. Given that PEDF has affinity for heparin, we examined binding to heparin by both forms. Heparin affinity column chromatography showed that both forms bound to heparin-conjugated beads with similar affinity (Figure 4(b)). Hyaluronan binding assays were performed with PEDF-1 and PEDF-2 (data not shown) and showed similar results. Altogether, the results indicate that there are no differences in the overall protein conformation and in binding affinity to glycosaminoglycans between the two PEDF forms.

3.4. Effects of PEDF Versions on Tumor Cell Viability. To compare the antitumorigenic effects of PEDF-1 and PEDF-2, T24, 231-BR, and 4T1-BR5 cell cultures were incubated with serum-free medium containing increasing concentrations of each PEDF form, and monitored, in parallel, in real time, for cell impedance using a microelectronic system and under the microscope. Cells treated without PEDF increased the electrical impedance represented as cell index value with time up to 72–96 h. However, the cell index decreased in those treated with PEDF-2 in a dose-dependent fashion, while this was not observed with PEDF-1 (Figure 5), suggesting a differential effect on lowering the cell number. Examination under the microscope revealed a decline in cell number with increasing PEDF-2 additions and not with PEDF-1 with the three types of cells (Figure 6). The numbers of cells with 100 nM PEDF-2 in each case were significantly decreased in each field relative to those without or with PEDF-1. Quantification of relative cell numbers using two different biomarkers for live cells, intracellular ATP content, and mitochondrial dehydrogenase activity at end point corroborated the observation that PEDF-2 decreased the viability of each cell type, while PEDF-1 did not have a significant effect (Figure 7). Altogether, treatment with PEDF-2 resulted in a dramatic direct negative effect in tumor cell viability in contrast to PEDF-1.

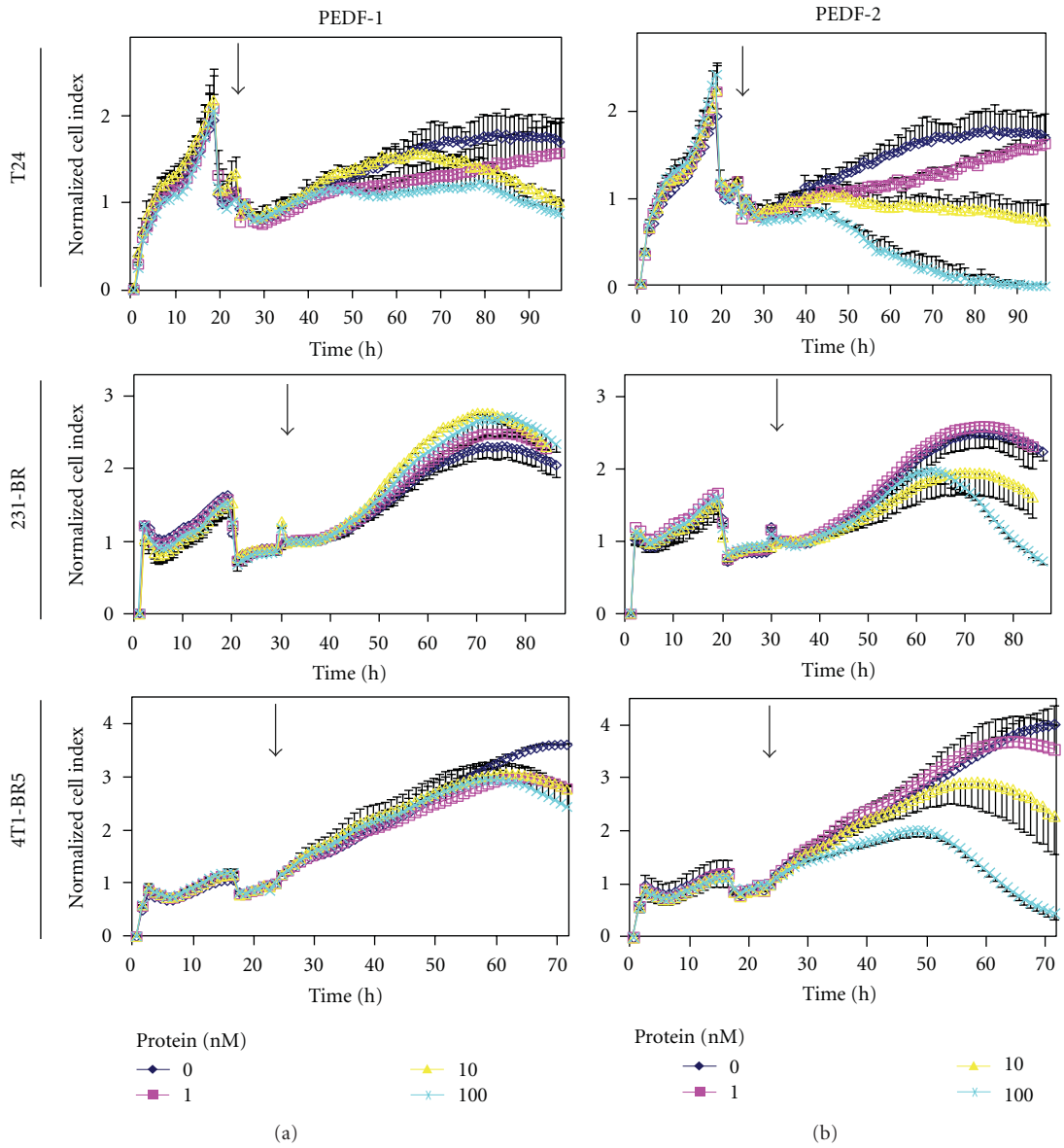

Figure 5: Effects of PEDF proteins on real-time electrical cell impedance of T24, 231-BR, and 4T1-BR5 cells in culture: cells were cultured in complete media to subconfluency, followed by serum starvation for 8 h, and then media was replaced with fresh media without serum containing indicated concentrations of recombinant human PEDF-1 or PEDF-2 proteins. Real-time cell impedance was monitored in cells treated with indicated concentrations of PEDF. Cell index was normalized relative to the cell numbers at the time of PEDF addition (shown by arrow). Each point is the average of triplicate assays. Error bars indicate average ± SD.

3.5. Effect of PEDF-1 and PEDF-2 on Cancer and RPE Cell Motility. It has been reported that PEDF inhibits the migration of breast tumor MDA-MB-231 cells and retinal pigment epithelial (RPE) cells [20, 27]. Thus, the effect of the PEDF-1 and PEDF-2 proteins on cell migration was examined in MDA-MB-231 cells and ARPE-19 cells. For this wound healing assay, a fixed-width scratch in a cell monolayer was created and the advancement of the migrating front was followed in the presence of either PEDF-1 or PEDF-2. PEDF-2 affected negatively the migration of the MDA-MB-231 cells, while PEDF-1 was not significant (Figure 8(a)). Wound healing assays performed with ARPE-19 cells showed similar results as with the cancer cells (Figure 8(b)). These observations indicate that PEDF-2 was more efficient than PEDF-1 in inhibiting cancer and RPE cell migration.

3.6. PEDF-1 Is Active on Retina R28 Cells against Serum-Starvation-Induced Death. The above results suggest that PEDF-2 is more efficient than PEDF-1, implying that PEDF-1 may have lost its activity. It has been reported that PEDF also protects retina cells against trophic factor depletion [28, 29]. Thus the activity of PEDF-1 was also examined using a method in which PEDF protects retina cells against death induced by serum starvation. Figure 9 shows cell viability of retina R28 cells treated with increasing amounts of PEDF in

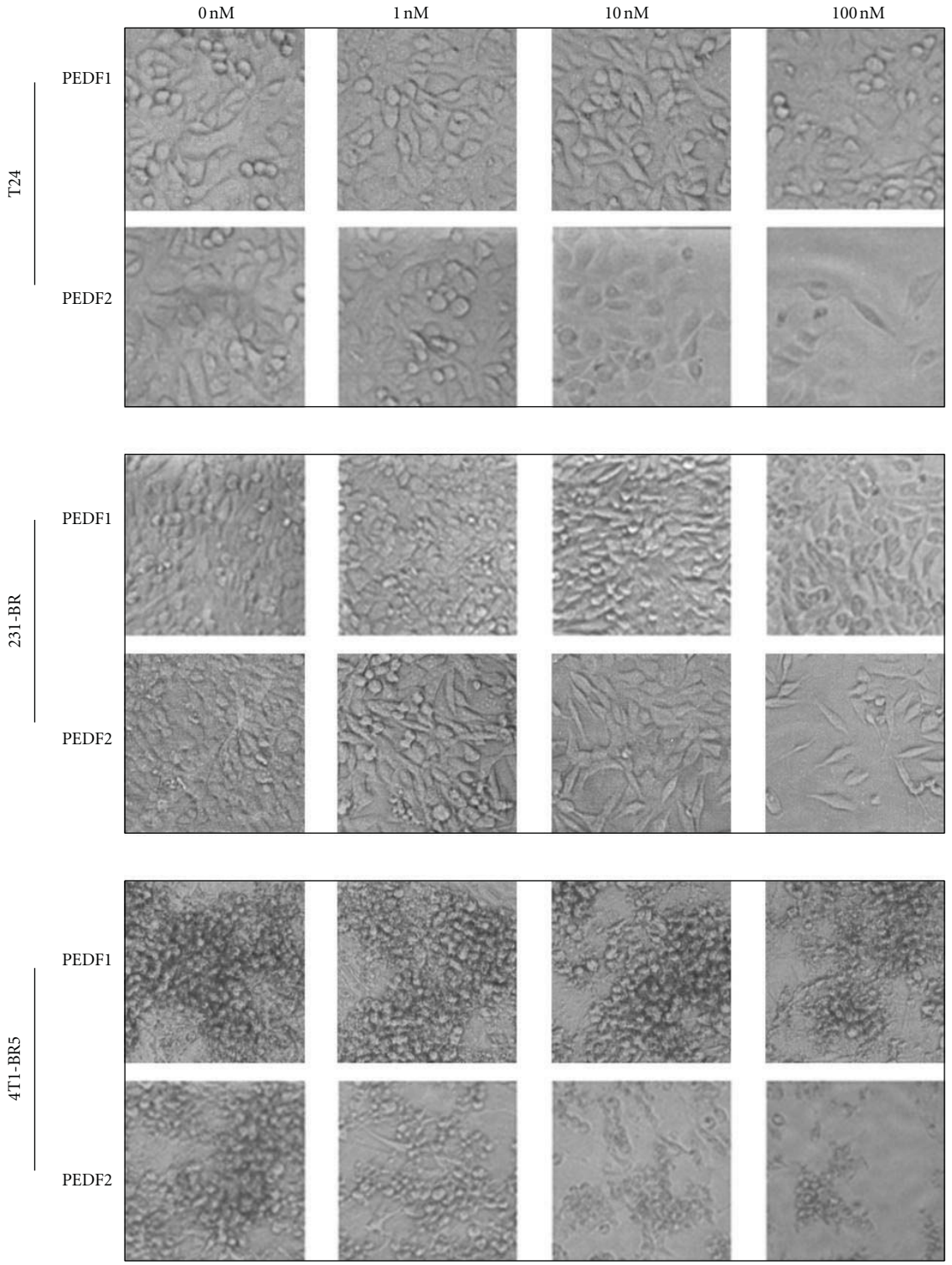

Figure 6: Imaging of T24, 231-BR, and 4T1-BR5 cells treated with PEDF-1 and PEDF-2 proteins: bright field images were taken at the end point of cells treated with PEDF proteins at concentrations indicated on the top.

serum-free media for 48 hours. Figure 9(a) shows that real-time cell index of R28 cells decreased almost linearly between the 20th hour and the 48th hour after plating. However treatments with increasing concentrations of PEDF-1 prevented the cell index decrease, which with the highest dose of PEDF (100 nM) even increased from the 20th to the 35th hour after plating. Quantification of relative cell numbers at end point using intracellular ATP content as biomarker of live cells corroborated the observation that PEDF-1 increased viability of R28 cells in a dose-dependent fashion (Figure 9(b)).

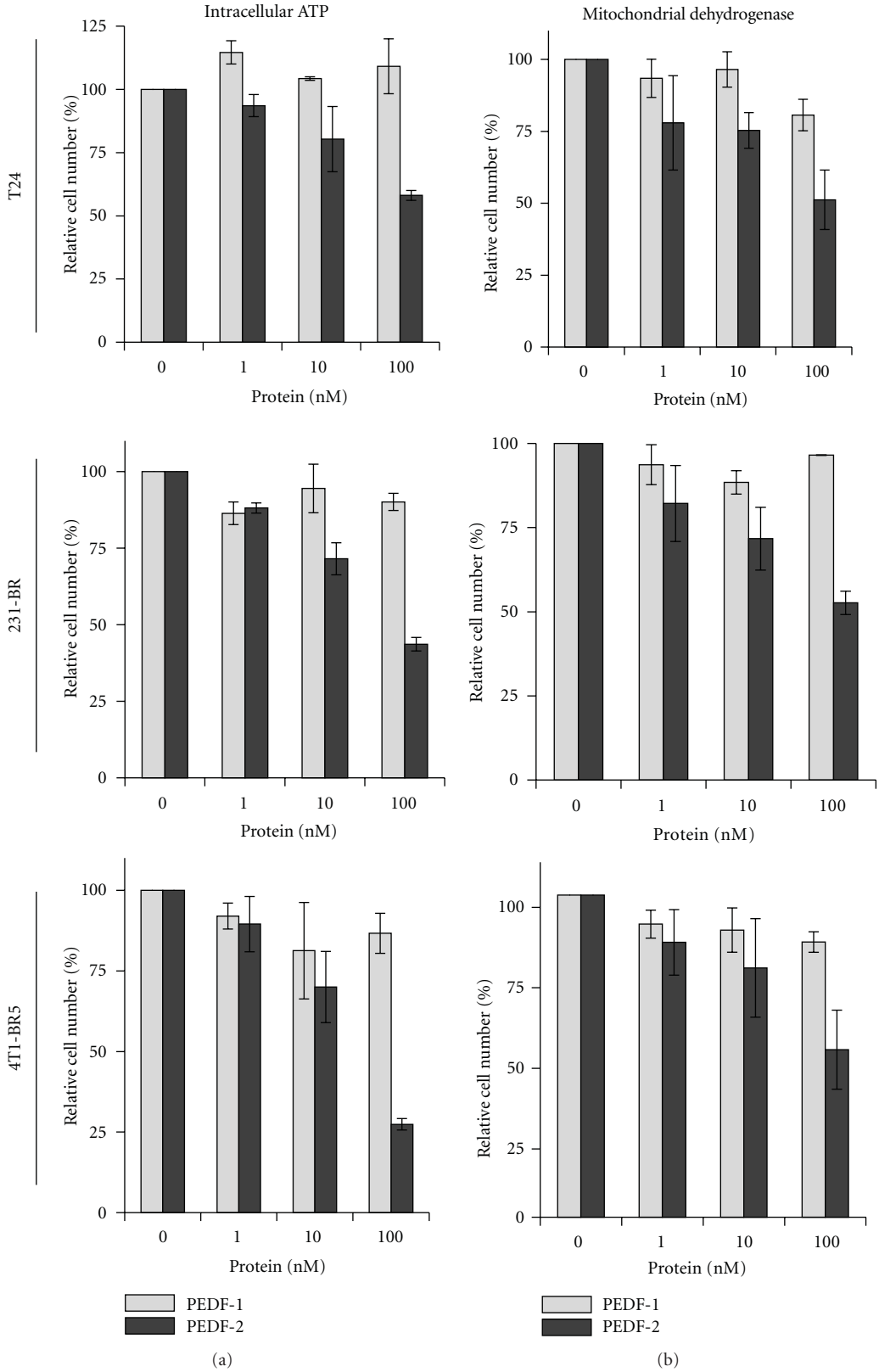

Figure 7: Effects of PEDF proteins on viability of T24, 231-BR, and 4T1-BR5 cells in culture: effect of PEDF on cell viability at the treatment end point. Plot of relative cell numbers quantified based on intracellular ATP content (shown on the left) and mitochondrial dehydrogenase activity (shown on the right) as biomarkers of live cells. Cell viability was expressed as percentage of luminescence values relative to untreated controls, and percentage of absorbance at 490 nm relative to untreated controls, respectively. Each point is the average of triplicate assays. Error bars indicate average ± SD.

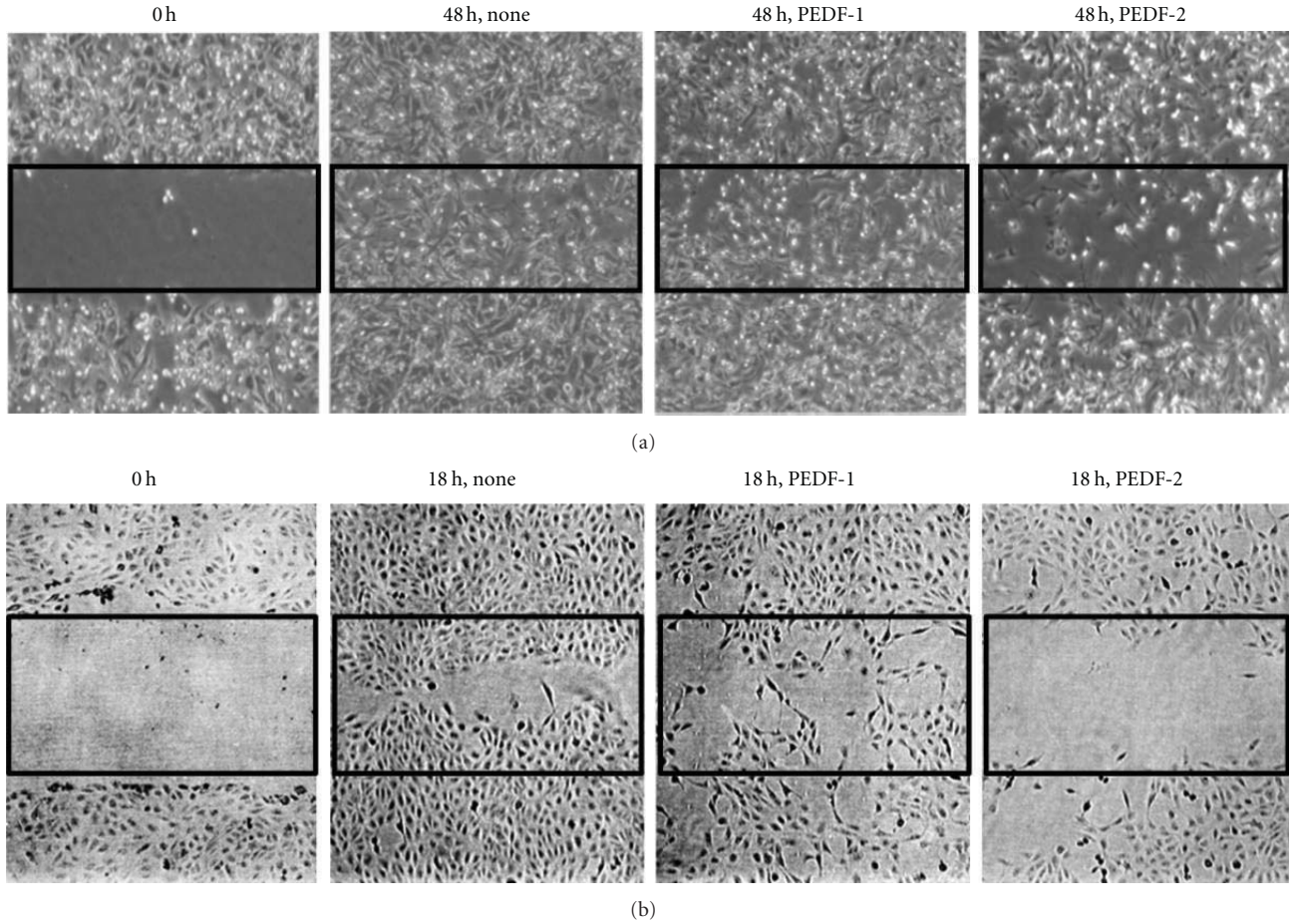

Figure 8: Effect of PEDF proteins on wound healing assay: MDA-MB-231 (a) and ARPE-19 (b) cell monolayers were scratched with a 10-μL pipette tip to make a wound. Cells were then incubated with media alone or PEDF proteins (20 ng/mL) for 48 h in (a) and 18 h in (b). Images were taken at the end points and compared to 0 h to measure wound healing.

The effects on the retina survival activity of PEDF-2 were similar to those of PEDF-1 (Figures 9(c) and 9(d)). Altogether, the results show that both PEDF forms PEDF-1 and PEDF-2 behaved as active survival factor for retina cells in culture.

4. Discussion

We have identified two distinct biochemical forms of recombinant PEDF, which we termed PEDF-1 and PEDF-2. The distinction is based on ionic potential, pI values, and apparent molecular weight and demonstrates that PEDF-2 has higher overall negative charge than PEDF-1. PEDF-2 elution required higher ionic potential from anion-exchange column chromatography; the protein has a lower pI and migrated slower in SDS-PAGE than PEDF-1. This characteristic is shared with PEDF from different sources and even when it is altered or fused to tags. Altered and FLAG-tagged PEDF yielded two peaks similar to the wild-type PEDF (Figure 2), confirming that the existence as two forms is a general feature of PEDF. Because the protein derives from one PEDF cDNA sequence transfected into BHK cells, the sequence of the polypeptide backbone is identical in both forms. Upon maturation and secretion, the PEDF versions may have adopted modifications posttranslationally that confer charge differences, and in turn grant diversity in biological activities to PEDF. Isoforms of PEDF have been described by different laboratories. Recombinant PEDF isoforms were reported to have differences in carbohydrate composition at its glycosylation site [18]. PEDF isoforms have also been reported in peripheral neuropathies, with fucose residues on the oligosaccharide chain found only in the isoforms downregulated in neuropathic patients without pain [30]. Although both PEDF-1 and PEDF-2 are glycosylated, they differ in apparent molecular weight even after deglycosylation, suggesting that this is not the main difference between them. More recently, it was reported that the triple phosphomimetic PEDF, EEE-PEDF, containing three negatively charged glutamic acids instead of serines, possesses significantly increased antiangiogenic, and anticancer activities than wild-type PEDF [20, 31]. However, our PEDF forms were not phosphorylated (data not shown). The significance of these findings is that PEDF isoforms

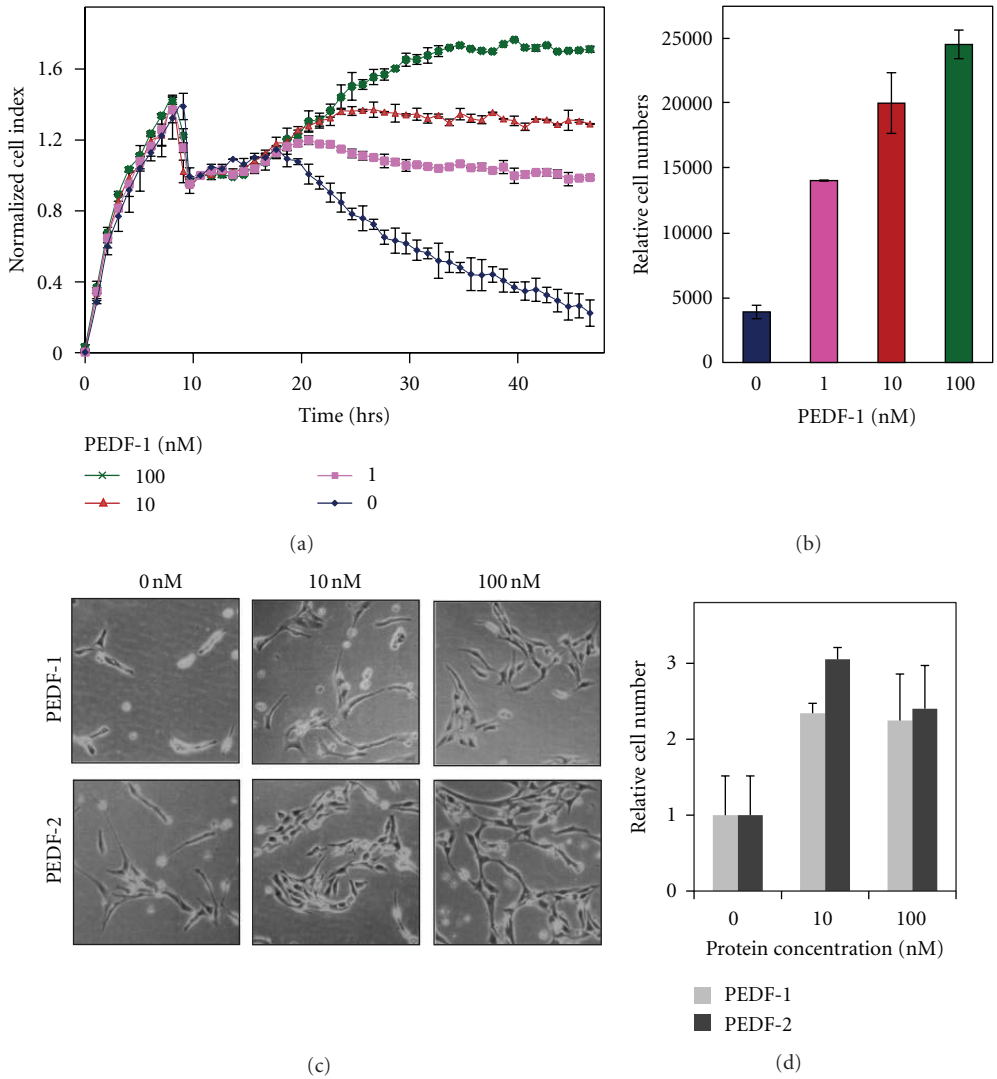

Figure 9: Retina R28 Cell Survival Assays: (a) real-time cell impedance monitoring of PEDF cell survival activity in serum-starved R28 cells in the presence of increasing concentrations of PEDF. The cell index was normalized relative to the cell numbers at the time of PEDF addition. (b) Plot of relative cell numbers quantified based on intracellular ATP content performed at end point (see (a)). (c) Bright field images of R28 cells were taken at end point (48 h) of cells treated with PEDF proteins at concentrations indicated on the top. (d) Plot of relative cell numbers quantified based on intracellular ATP content performed at 48 h. Each point is the average of duplicate wells. Error bars indicate average ± SD.

have been identified in ocular, tumor, and nontumor tissues [16, 17, 32], and interestingly their distribution varies between normal and tumor tissues.

Several reports on posttranslational modifications of PEDF offer explanations for the multifunctional modality of PEDF. Similar to our results, Duh et al. [18] purified two forms of PEDF, A and B, using cation-exchange column. Form A eluted at a lower NaCl concentration, that is, less positively charged and had a slightly larger molecular weight than from B; and PEDF A was a more efficient antiangiogenic form than PEDF B. This implies a match of PEDF-1 to PEDF B and PEDF-2 to PEDF A. Petersen et al. [33] reported that a complete map of all post-translational modifications revealed that authentic plasma PEDF carries an N-terminal pyroglutamate blocking group and an N-linked glycan at position Asn266. They proposed that the pyroglutamate residue may regulate the activity of PEDF analogously to the manner in which it regulates thyrotropin-releasing hormone. Maik-Rachline et al. [34] demonstrated that extracellular phosphorylation converts PEDF from a neurotrophic to an antiangiogenic factor. Later they proposed that differential phosphorylation induces variable effects of PEDF, and therefore contributes to the complexity of PEDF action [19]. Although the observations with PEDF phosphomimetics match our results, our PEDF forms were not found to be phosphorylated and therefore, another chemical difference is sought for PEDF-1 and PEDF-2 that is yet to be determined. These observations are in agreement with the idea that the

multifunctional modality of PEDF may be explained by differences in posttranslational modifications of the PEDF polypeptide, which may regulate their biological activities. Given that synthetic peptides 34-mer and 44-mer derived from PEDF are biologically active, these regions may require to be properly exposed in the folded protein to interact with cell surface receptors and trigger the necessary signals for activity. Posttranslational modifications may be tools to open up or expose the active regions in the folded PEDF protein. It is envisioned that in PEDF-2 the active region for antitumorigenic activity is more exposed than in PEDF-1. Other factors that enhance efficacy in PEDF-2 may include post-translational modification(s) that confers an increase in negative charge to the protein. Further studies to identify the chemical differences between the PEDF forms will be of great interest for the development of second generation PEDF molecules.

Authors' Contribution

P. Subramanian and M. Deshpande share first coauthorship and they contributed equally to this paper.

Acknowledgments

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Research Article

Efficacy of Continuously Administered PEDF-Derived Synthetic Peptides against Osteosarcoma Growth and Metastasis

Matthew L. Broadhead,[1] Peter F. M. Choong,[1,2] and Crispin R. Dass[3]

[1] *Department of Orthopaedics and Department of Surgery, St. Vincent's Hospital, University of Melbourne, Fitzroy VIC 3065, Australia*
[2] *Sarcoma Service, Peter MacCallum Cancer Centre, East Melbourne, VIC 3002, Australia*
[3] *School of Biomedical and Health Sciences, Victoria University, St. Albans, VIC 3021, Australia*

Correspondence should be addressed to Matthew L. Broadhead, matthew.broadhead@gmail.com

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The potent antiangiogenic pigment epithelium-derived factor (PEDF) has shown promise against osteosarcoma, a tumour that originates in the bone and metastasises to the lungs. Neurotrophic, antiangiogenic, antiproliferative, and antimetastatic properties of PEDF have been attributed to a number of functional epitopes on the PEDF glycoprotein. StVOrth-2 (residues 78–102) and StVOrth-3 (residues 90–114) are two PEDF-derived peptides based on these functional epitopes. StVOrth-2 has previously been shown to inhibit osteosarcoma cell proliferation, while StVOrth-3 increased osteosarcoma cell adhesion to collagen I *in vitro*. In this paper, we have evaluated systemically and continuously delivered StVOrth-2 and StVOrth-3 using a clinically relevant murine model of osteosarcoma with spontaneous metastasis. Treatment with StVOrth-2 or StVOrth-3 with microosmotic pumps was initiated after primary osteosarcoma was established in the tibia. While treatment with StVOrth-2 and StVOrth-3 did not appear to affect local tumour invasion, tumour necrosis or apoptosis, StVOrth-2 predominantly restricted the growth of primary tumours, while StVOrth-3 restricted the burden of pulmonary metastatic disease. No peptide caused gross toxicity in mouse tissues as assessed by measuring weight of animals, serum biochemistry, and gross tissue observation. The differential effects exhibited by StVOrth-2 and StVOrth-3 in this orthotopic model of osteosarcoma may be related to the functional epitopes on the PEDF glycoprotein that they represent.

1. Introduction

Pigment epithelium-derived factor (PEDF) is a 50 kDa endogenous glycoprotein that was first discovered in 1991 as a factor secreted by the pigment epithelium of the human foetal eye [1]. PEDF was shown to promote differentiation of retinoblastoma cells [2] and was implicated in a range of eye pathologies including diabetic retinopathy, macular degeneration, and retinitis pigmentosa and glaucoma [3]. PEDF is a potent antiangiogenic agent, more potent than angiostatin, endostatin and thrombospondin-1 by endothelial cell migration assay [4]. PEDF has also been shown to be an antitumorigenic agent for malignancies including osteosarcoma, melanoma, glioma, lung, breast, prostatic, ovarian and pancreatic carcinomas [5].

Biochemical studies have enabled the identification of multiple functional epitopes for PEDF. The interactions between these epitopes and receptors are likely to initiate divergent signalling pathways for the different cellular effects of PEDF. Filleur et al. [6] first characterised 34-mer (residues 24–57) and 44-mer (residues 58–101) PEDF-derived peptides that conferred antiangiogenic and neurotrophic activity, respectively. Additionally, another sequence, ERT (residues 79–94), showed both antiangiogenic and differentiation activity. The properties of these peptides were demonstrated *in vitro* by endothelial cell apoptosis and chemotaxis assays and Y-79 retinoblastoma differentiation assay. Using a subcutaneous tumour model and PC-3 prostate cancer cells, expression of the 34 mer peptide reduced tumour microvascular density and induced tumour cell apoptosis, effects not demonstrated for the 44-mer peptide [6]. The 34 mer peptide restricted angiogenesis through a c-jun-NH2 kinase (JNK-) dependent pathway leading to NFATc2 deactivation and c-Flip antagonism [6].

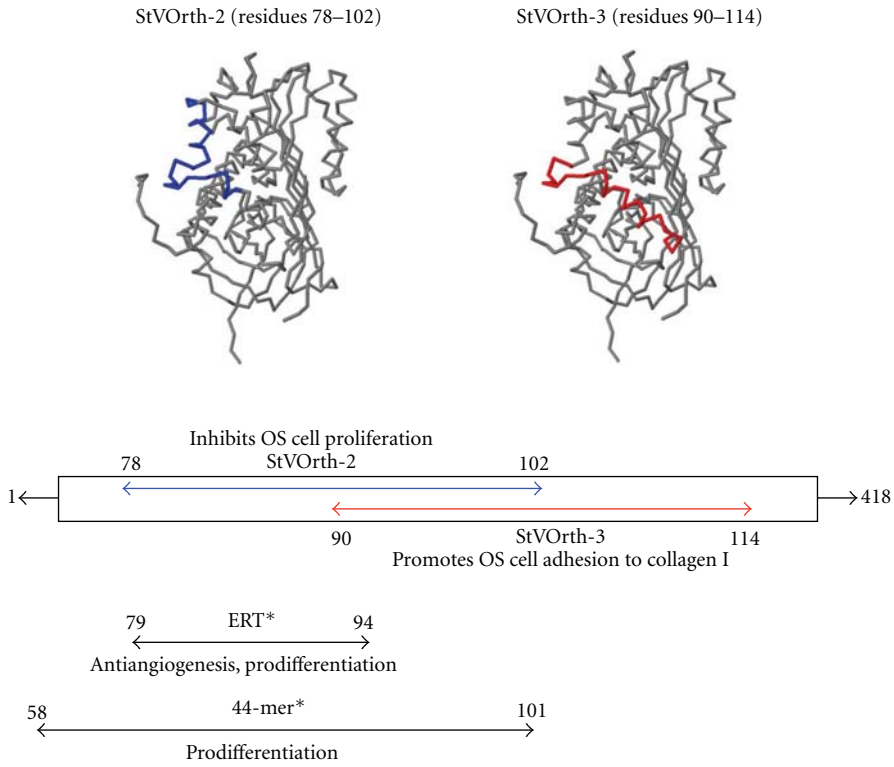

Figure 1: StVOrth-2 and StVOrth-3 peptides. StVOrth-2 (highlighted in blue) consists of residues 78–102 of the parent PEDF sequence. StVOrth-3 (highlighted in red) consists of residues 90–114. StVOrth-2 and StVOrth-3 have previously been shown to inhibit osteosarcoma cell proliferation and promote osteosarcoma cell adhesion to collagen I *in vitro*, respectively [7]. *ERT and 44-mer peptide sequences, as described by Filleur et al. [6], overlap with the StVOrth-2 and StVOrth-3 sequences.

In another study, four different PEDF-derived peptides, termed StVOrth-1, StVOrth-2, StVOrth-3, and StVOrth-4, consisting of PEDF residues 40–64, 78–102, 90–114, and 387–411, respectively, were tested *in vitro* and *in vivo* [7]. *In vitro*, StVOrth-2 was the most potent inhibitor of SaOS-2 osteosarcoma cell proliferation, while StVOrth-3 dramatically promoted SaOS-2 adhesion to collagen I. StVOrth-4 inhibited SaOS-2 cell invasion through Matrigel. StVOrth-1, -2, and -3 all induced osteoblastic differentiation. StVOrth-3 and StVOrth-4 reduced VEGF expression in SaOS-2 osteosarcoma cells. StVOrth-2 and StVOrth-3 were then evaluated *in vivo* using an orthotopic murine model of osteosarcoma. Notably, StVOrth-2 (residues 78–102) and StVOrth-3 (residues 90–114) possessed sequences that overlapped with the 44-mer (residues 58–101) and ERT (residues 79–94) sequences described by Filleur et al. [6] (Figure 1). Both StVOrth-2 and StVOrth-3 restricted osteosarcoma tumour growth and inhibited the development of pulmonary metastases when SaOS-2 cells were treated prior to intratibial injection.

The findings of both Filleur et al. [6] and Ek et al. [7] provide some insight into how PEDF structure relates to its multidimensional ability to restrict tumour progression. However, the study design and methods of peptide delivery used in these models make it difficult to extrapolate the findings for human use. Filleur et al. [6] used a subcutaneous tumour model with transfected PC-3 cells to demonstrate the differential effects of the 34-mer and 44-mer PEDF-derived peptides. Gene therapies have yet to be proven safe for human application, making it unlikely that they will be used for osteosarcoma therapy in the near future [8]. Ek et al. [7] used the SaOS-2 osteosarcoma cell line to achieve a spontaneously metastasizing murine model of orthotopic osteosarcoma. SaOS-2 cells were treated with StVOrth-2 and StVOrth-3 peptides prior to intraosseous injection, thus facilitating early uptake of peptides and change in phenotype. In order to evaluate the true efficacy of PEDF-derived peptides against established osteosarcoma, treatment should be delayed until after the establishment of primary tumours. This would better simulate the clinical presentation and treatment of osteosarcoma in humans.

2. Materials and Methods

2.1. Cells and Culture Conditions. The SaOS-2 human osteosarcoma cell line (American Tissue Culture Collection, Manassas, VA, USA) was cultured in complete medium, CM, at 37°C and in humidified 5% CO_2. CM consisted of MEM-Alpha+GlutaMAX (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA, USA). Exponentially growing cells, with passage number always less than 20, were used for the studies.

2.2. Establishment of the Orthotopic Model of Osteosarcoma. 5-week-old Balb/c nude mice were purchased from the Animal Resource Centre, Perth, Australia and were housed at the St Vincent's Hospital BioResources Centre under PC2 pathogen-free conditions. Animal ethics approval was obtained from the St Vincent's Hospital Melbourne Animal Ethics Committee. A 50% concentration of Matrigel was used to dilute SaOS-2 osteosarcoma cells to a concentration of 2×10^6 cells/mL. Following anaesthesia with intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg), a 27-gauge needle was introduced into the left tibia of each mouse using a gentle drilling motion in order to avoid iatrogenic fracture [9], and a volume of 10 μL of SaOS-2/Matrigel solution was injected. Postinjection, the needle was retracted slowly to prevent backflow of injectate.

Tumour growth and animal weights were monitored twice weekly until the endpoint of the study. Anteroposterior (AP) and lateral (L) dimensions of limbs were recorded using digital callipers. Volumes were calculated from these dimensions using the formula $4/3\pi[1/4(\mathrm{AP}+\mathrm{L})]^2$ [7]. The contralateral nontumour-bearing limb was used as a control to calculate actual tumour volume.

Primary orthotopic tumours were apparent at day 20 after SaOS-2 inoculation, when average tumour volume was 22.5 mm^3, at which point mice were randomised into treatment groups each consisting of four mice. As outlined below, these groups received either: (1) sterile water as control, (2) StVOrth-2 at 50 μg/kg/day, (3) StVOrth-2 at 500 μg/kg/day, (4) StVOrth-3 at 50 μg/kg/day, or (5) StVOrth-3 at 500 μg/kg/day.

2.3. Delivery of PEDF-Derived Synthetic Peptides. PEDF-derived peptides, StVOrth-2 (residues 78–102) and StVOrth-3 (90–114), were designed and sourced previously according to the procedure outlined by Ek et al. [7]. This paper refers to the full-length human PEDF sequence, and amino acid numbering is based on those sequences listed in GenBank (National Institutes of Health). StVOrth-2 is the aminoacid sequence $_{78}$VLLSP LSVAT ALSAL SLGAE QRTES$_{102}$. StVOrth-3 is the sequence $_{90}$SALSL GAEQR TESII HRALY YDLIS$_{114}$. High-performance liquid chromatography (HPLC) and mass spectrometry was used to confirm the purity of these peptides. Ek et al. [7] showed that StVOrth-2 inhibited SaOS-2 osteosarcoma cell proliferation, while StVOrth-3 inhibited SaOS-2 osteosarcoma cell adhesion to collagen I *in vitro*. StVOrth-2 and StVOrth-3 sequences overlap with the 44-mer (residues 58–101), and ERT (residues 79–94) sequences described by Filleur et al. [6] (Figure 1).

Sustained delivery of StVOrth-2, StVOrth-3, or sterile water (negative control, placebo) was achieved by intraperitoneally implanted Alzet microosmotic pump (Durect Corp., Cupertino, CA, USA). Pumps were aseptically filled with the different treatments and surgically implanted within the peritoneal cavity of animals for systemic delivery. This surgery was performed at day 20 after SaOS-2 injection. The mean pumping rate for the Alzet microosmotic pump (model 1002) is 0.25 μL/hr over 14 days, as determined by the manufacturer. StVOrth-2 and StVOrth-3 were administered at 50 μg/kg and 500 μg/kg daily doses. Sterile water was used as diluent for StVOrth-2 and StVOrth-3.

The human physiological serum concentration of PEDF ranges between 4 ng/mL (80 pM) to 15 μg/mL (300 nM) [10–15]. In one study, inhibition of vessel formation in ischemia-induced retinopathy was achieved at a 50 nM concentration [16]. The 50 μg/kg and 500 μg/kg doses used in the present study are equivalent to 1 μg/mL (20 nM) and 10 μg/mL (200 nM) concentrations of PEDF, respectively, which lies within the range mentioned above. This assumes an average mouse weight of 20 grams and an average blood volume of 1 mL [17]. It is known that the pumps are capable of delivering a steady state quantity of peptides into the abdominal cavity [18, 19], which will eventually be taken into the microvasculature supplying the abdominal region.

2.4. Study Endpoint and Tissue Analysis. Tumours had grown to a disabling size for control animals at day 34 after SaOS-2 inoculation as expected. This was the humane endpoint of the study, and all animals were euthanized under anaesthesia by cervical dislocation at this time. Following this, tumour-bearing limbs, lungs, hearts, small intestines, and skin were harvested for examination. All specimens were fixed in 4% paraformaldehyde, followed by paraffin embedding. Blood samples were obtained after cervical dislocation and dissection through the thoracic cage and were immediately treated with anticoagulants. Affected limbs were X rayed at 35 kV for 30 s using a cabinet system (Faxitron Corp., Wheeling, IL, USA).

Blood collected after euthanasia was analysed for renal and hepatic biochemical parameters (serum creatinine, alanine transaminase (ALT), and aspartate transferase (AST)) using a Sysmex XE2100 instrument [20]. Tissues were embedded in paraffin prior to histological preparation and analysis. For preparation of paraffin sections, 4 μm sections of tumours and viscera were cut by microtome. Tumours were sectioned to provide an *en face* surface for the quantification of per cent tumour necrosis and apoptosis.

Primary tumours, lungs, heart, small intestine, and skin sections were dehydrated through an ethanol series followed by xylene, then stained with haematoxylin and eosin. A terminal dUTP nick end labelling (TUNEL) assay kit (Promega, Madison, WI, USA) was used to detect apoptotic cells in primary tumours [21]. Pertex mounting agent was used to seal coverslips to slide sections, and all tissues were observed using a Nikon Eclipse TE2000-U microscope (Nikon, Lidcombe, NSW, Australia) and photographed with SPOT Advanced software (SciTech, Aurora, IL, USA).

The effect of intraperitoneal StVOrth-2 and StVOrth-3 on the development of pulmonary metastatic disease was examined histologically. Lungs were sectioned to achieve the greatest cross-sectional area and stained with haematoxylin and eosin. Micrometastases were identified by systematically scanning lung sections under 20x magnification. Discernible clusters of metastatic cells at this magnification were counted as micrometastases. Ten metastatic lesions were then randomly selected from each treatment group for measurement of cross-sectional areas.

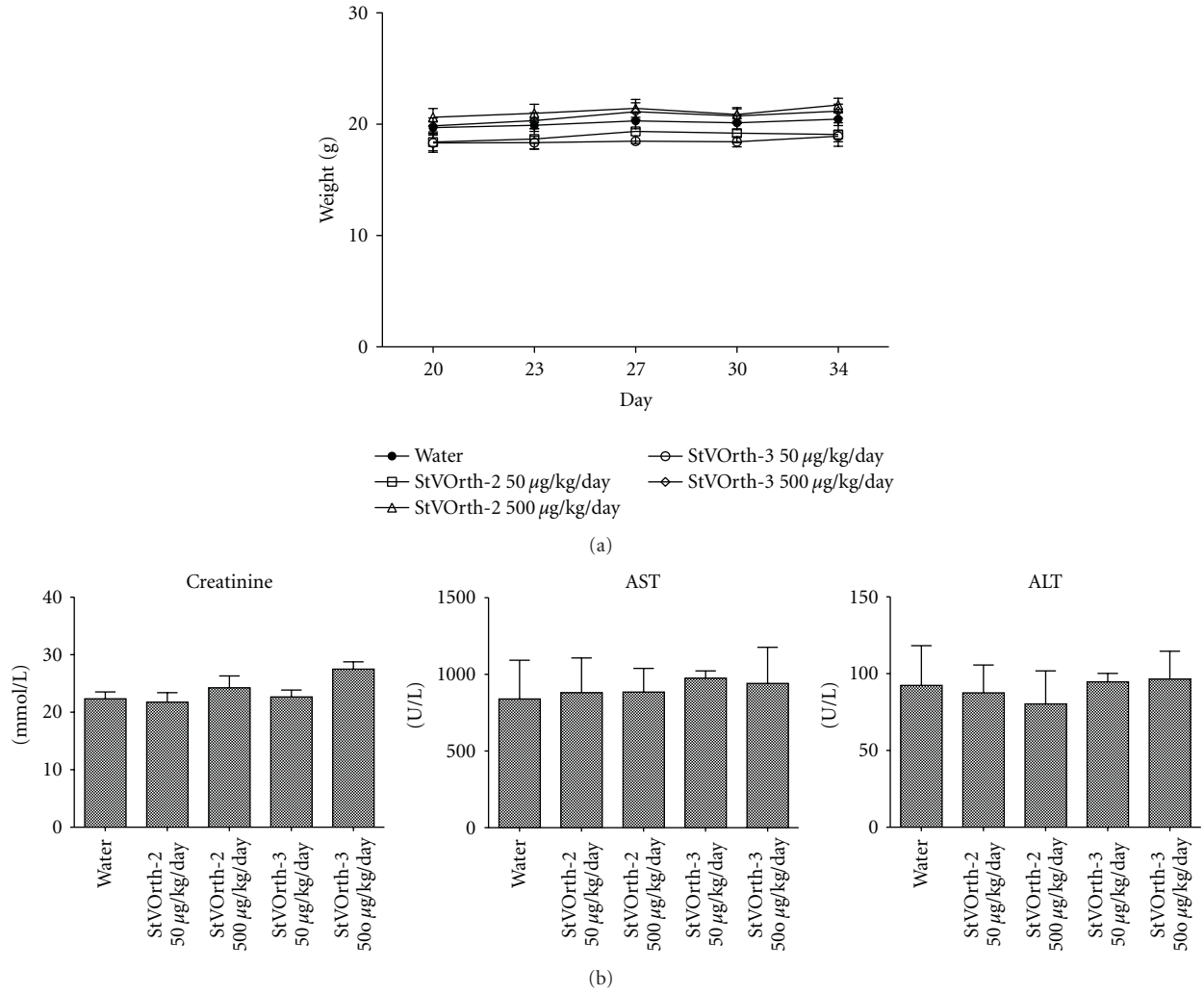

Figure 2: (a) Animal weights recorded at days 20, 23, 27, 30, and 34 after SaOS-2 intratibial injection. There was no significant weight loss compared to control. (b) Biochemical analysis of serum collected postmortem (±SEM). There was no evidence of renal or hepatic toxicity.

2.5. Statistical Methods and Imaging Software. One-way and two-way ANOVA analyses with Bonferroni multiple comparisons test were used where appropriate (GraphPad Prism 5 for Mac OS X). ImageJ (Version 1.45j, National Institutes of Health, USA) was used for all image analysis. Means and standard errors were calculated for all data collected.

3. Results

3.1. Potential Systemic Side Effects of PEDF-Derived Peptides. Mice were studied for potential systemic side affects associated with delivery of StVOrth-2 (residues 78–102) and StVOrth-3 (residues 90–114) (see Figure 1). Mice were regularly monitored during the study for signs of distress and animal weights were recorded twice weekly. All mice remained well for the duration of the study, and no significant weight loss compared to control was observed (two-way ANOVA) (Figure 2(a)).

Serum, hearts, lungs, skin, and small intestines were collected postmortem for histological examination. No features of cytotoxicity [22] were evident in these tissues, in either control or peptide-treated groups. Renal and hepatic serum biochemistry parameters were within physiological limits [23] for all treatment groups (Figure 2(b)).

3.2. Systemically Delivered StVOrth-2 Inhibits Osteosarcoma Tumour Growth. Orthotopic osteosarcoma in this murine model was treated with systemically delivered StVOrth-2 and StVOrth-3. Microosmotic pumps, continuously delivering StVOrth-2 and StVOrth-3 at daily doses of 50 μg/kg and 500 μg/kg, were implanted in the peritoneal cavities of mice at day 20 of the study. Tumours were well established at this time with an average tumour volume of 22.5 mm^3 (±1.7 SEM).

Sustained delivery of StVOrth-2 at both doses caused a significant reduction in primary tumour volume at the study endpoint. 50 μg/kg and 500 μg/kg StVOrth-2 caused 49.7% and 50.9% reductions in tumour volume, respectively, at day 34 ($P < 0.01$, two-way ANOVA with Bonferroni multiple comparisons test) (Figures 3(a) and 3(b)). A significant effect

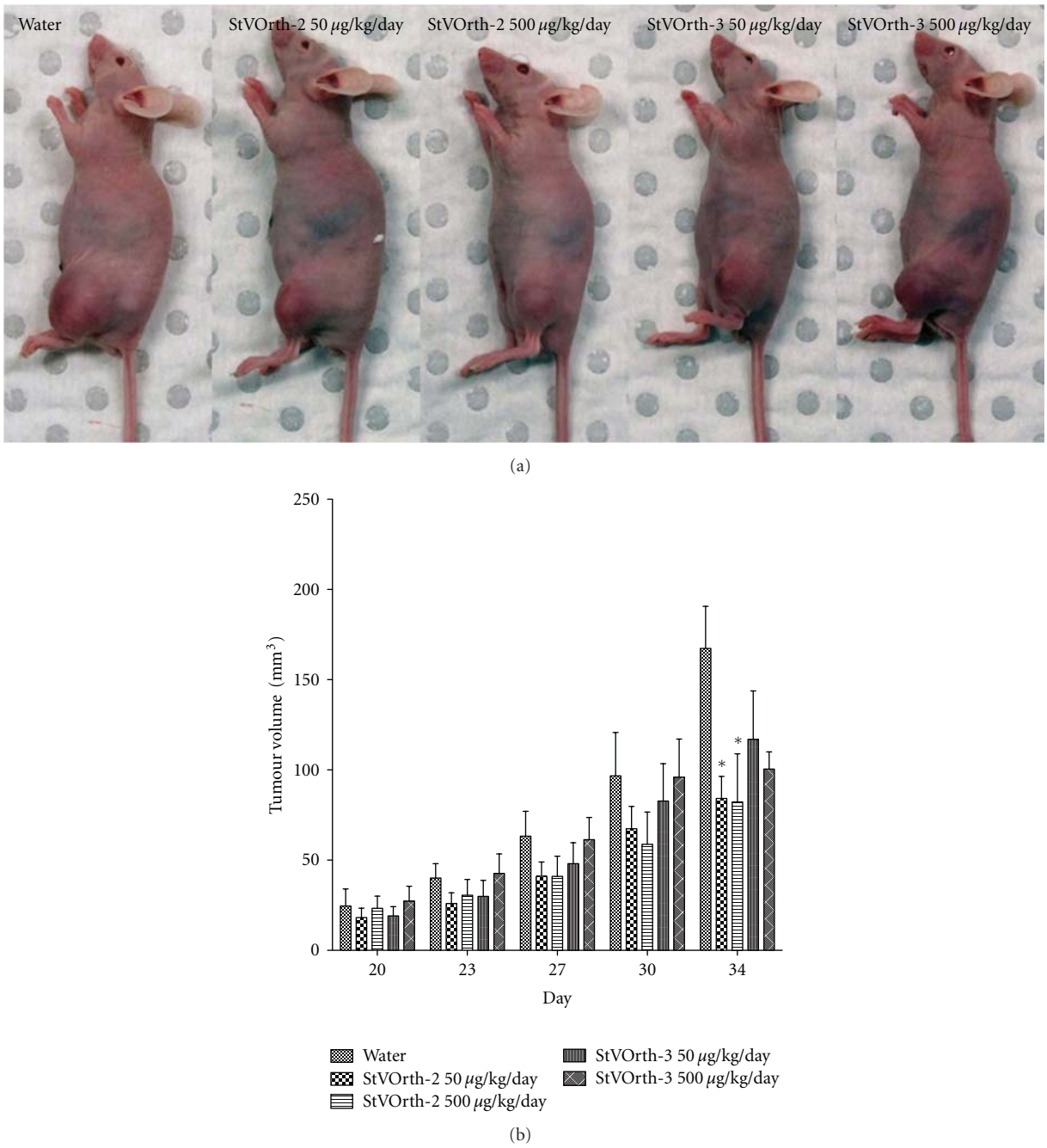

Figure 3: Systemically delivered StVOrth-2 inhibits growth of orthotopic osteosarcoma. (a) Photomicrographs of mice showing left tumour-bearing hindlimbs. (b) Tumour volumes recorded at days 20, 23, 27, 30, and 34 after SaOS-2 intratibial injection (±SEM). $^*P < 0.01$, two-way ANOVA.

was not seen before the day 34 time point, and there was no statistical difference between the two doses of StVOrth-2.

By comparison, the therapeutic effect of StVOrth-2 on primary tumour volume was unable to be replicated by systemic StVOrth-3 therapy. Despite achieving 30.1% and 40.0% reductions in primary tumour volume at day 34 with 50 μg/kg and 500 μg/kg StVOrth-3, respectively, these results did not achieve statistical significance (two-way ANOVA) (Figures 3(a) and 3(b)).

3.3. Tumour Invasion, Necrosis, and Apoptosis. Plain radiographs obtained after disarticulation of tumour-bearing limbs showed extensive soft tissue invasion and osteolysis for all animals (Figure 4). Invasion of surrounding structures, tumour necrosis, and apoptosis were assessed histologically. Treatment with either StVOrth-2 or StVOrth-3, at both 50 μg/kg and 500 μg/kg doses, did not affect orthotopic tumour invasion of the surrounding structures. All tumour-bearing animals showed invasion of skeletal muscles and

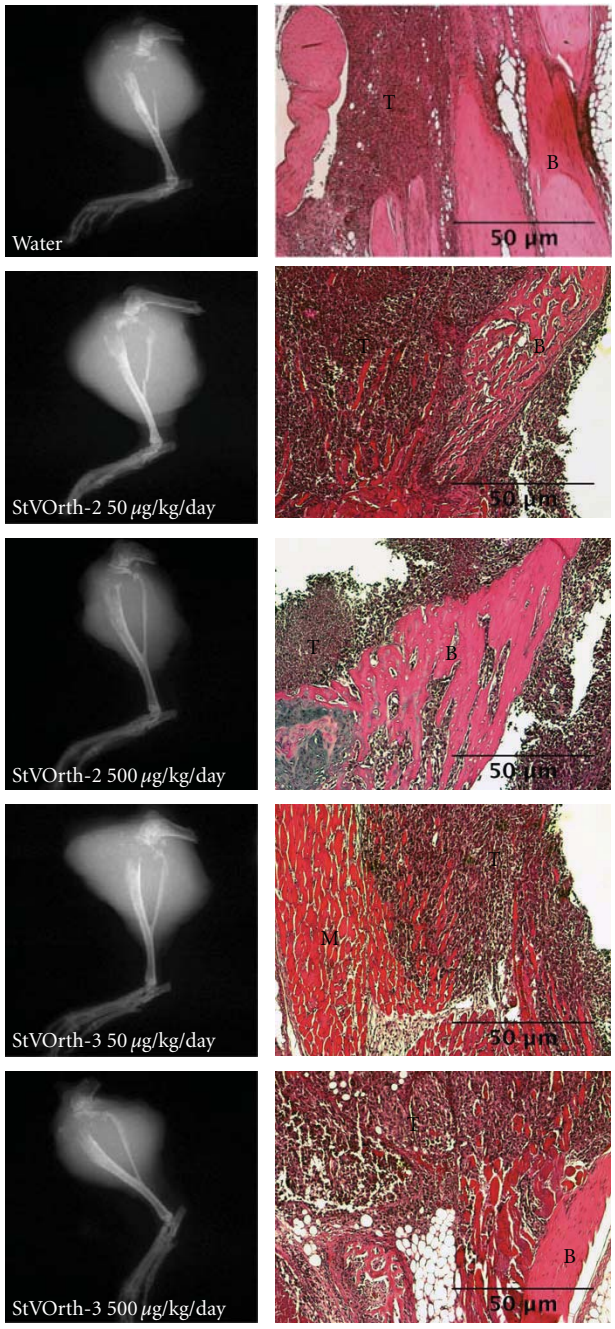

Figure 4: Tumour invasion. Plain radiographs (left) of tumour-bearing limbs show extensive osteolysis of proximal tibiae and soft tissue extension for all treatment groups. Haematoxylin and eosin-stained sections of orthotopic tumour (right) show tumour cells (T) invading bone (B) and skeletal muscle (M).

osteolysis on both sides of the adjacent joint. Tumour cells were clearly seen breaching the epiphyseal cartilage (Figure 4).

Treatment with StVOrth-2 or StVOrth-3 did not affect the degree of tumour necrosis. Per cent tumour necrosis was statistically equivalent for both StVOrth-2 and StVOrth-3 at either dose (one-way ANOVA). Similarly, treatment with StVOrth-2 or StVOrth-3 did not appear to affect tumour cell apoptosis. The percentage of TUNEL-positive staining tumour was found to be statistically equivalent across treatment groups (one-way ANOVA) (Table 1).

3.4. StVOrth-3 Restricts the Burden of Pulmonary Metastatic Disease. This SaOS-2 orthotopic model of osteosarcoma gives rise to spontaneous pulmonary metastases, and the burden of metastatic disease was assessed histologically post-mortem. Haematoxylin- and eosin-stained sections of lung tissue were examined under 20x objective. The numbers of micrometastases per lung section were enumerated, and treatment with StVOrth-2 or StVOrth-3, at both 50 μg/kg and 500 μg/kg doses, had no effect on the number of lesions observed (one-way ANOVA) (Figures 5(a) and 5(b)).

Treatment with StVOrth-3 did, however, cause a significant reduction in the size of micrometastatic lesions. The mean cross-sectional area of a micrometastasis was 0.19 mm^2 and 0.29 mm^2 for 50 μg/kg and 500 μg/kg StVOrth-3. This represented a 79% and 68.2% reduction in area compared to animals that received sterile water as control ($P < 0.05$, one-way ANOVA analysis with Bonferroni multiple comparisons test). Treatment with 50 μg/kg and 500 μg/kg StVOrth-2 resulted in mean cross-sectional areas of 0.68 mm^2 and 1.02 mm^2 respectively. These results, however, were not statistically significant (Figure 5(c)).

4. Discussion

Ek et al. [7] evaluated four PEDF-derived peptides based on the known functional epitopes of the PEDF glycoprotein. StVOrth-2 (residues 78–102) predominantly restricted SaOS-2 osteosarcoma cell proliferation and StVOrth-3 (residues 90–114) increased adhesion to collagen I. Both peptides restricted growth of primary osteosarcoma and the development of pulmonary metastases *in vivo* [7]. In this study we aimed to assess systemically delivered StVOrth-2 and StVOrth-3 as treatments for established osteosarcoma (both primary and secondary disease) and to evaluate the therapeutic safety of these peptides. We utilised the same orthotopic murine model of osteosarcoma described by Ek et al. [7] and delivered the StVOrth-2 and StVOrth-3 peptides systemically via an intraperitoneal microosmotic pump. Treatment was delayed until after the macroscopic appearance of primary osteosarcoma. Using this optimised model, differential effects on tumour growth and metastasis were demonstrated for StVOrth-2 and StVOrth-3, respectively.

Systemic delivery of StVOrth-2 restricted the volume of primary osteosarcoma. We observed 49.7% and 50.9% reductions in tumour volume with 50 μg/kg and 500 μg/kg StVOrth-2 treatments, respectively. Ek et al. [7] showed a reduction of more then 30% when these peptides were administered prior to SaOS-2 inoculation. It is interesting that StVOrth-2 caused greater inhibition of tumour growth than StVOrth-3. Ek et al. [7] showed that StVOrth-2 had a significant antiproliferative effect on SaOS-2 cells *in vitro*, while StVOrth-3 did not exhibit this effect. The

Table 1: Tumour per cent necrosis and apoptosis as determined on haematoxylin and eosin-stained and TUNEL-stained sections of orthotopic tumour, respectively.

	Water	StVOrth-2 50 μg/kg/day	StVOrth-2 500 μg/kg/day	StVOrth-3 50 μg/kg/day	StVOrth-3 500 μg/kg/day
% Necrosis	28.2% (±8.01 SEM)	31.3% (±11.85 SEM) $P > 0.05$	8.9% (±2.952 SEM) $P > 0.05$	46.6% (±10.04 SEM) $P > 0.05$	33.5% (±4.071 SEM) $P > 0.05$
% Apoptosis	20.2% (±2.22 SEM)	15.9% (±1.87 SEM) $P > 0.05$	3.6% (±0.56 SEM) $P > 0.05$	39.4% (±13.89 SEM) $P > 0.05$	41.3% (±10.12 SEM) $P > 0.05$

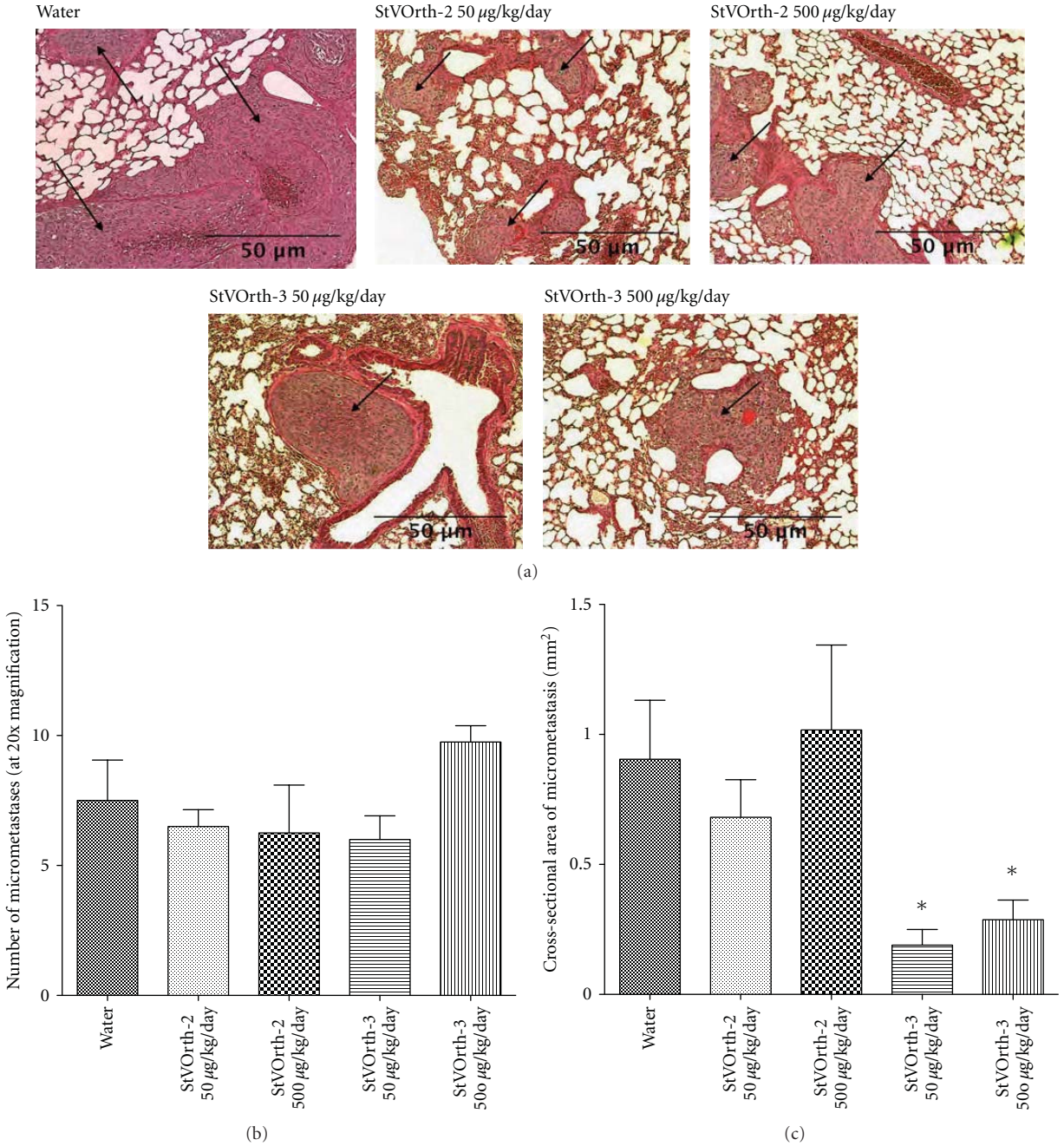

Figure 5: Systemically delivered StVOrth-3 inhibits progression of pulmonary metastatic disease. (a) Haematoxylin- and eosin-stained sections of lung tissue show micrometastases (arrows) disrupting the native alveolar architecture. Metastatic lesions were predominately subpleural and around larger airways. (b) Number of pulmonary micrometastases per lung section at 20x magnification (±SEM). (c) Cross-sectional areas of pulmonary micrometastases (±SEM). $^*P < 0.05$, one-way ANOVA.

molecular mechanisms exploited by StVOrth-2 to achieve its antitumour effect are yet to be characterised.

Treatment with StVOrth-2 and StVOrth-3 did not appear to affect local tumour invasion, tumour necrosis, or apoptosis. Tumour cells were observed invading the soft tissues and destroying the local bony architecture in all animals, independent of treatment type. Although groups receiving StVOrth-2 and StVOrth-3 were unable to be statistically differentiated based on tumour cell necrosis and apoptosis, it was evident that the percentages of tumour necrosis and apoptosis were proportional to tumour volume. This SaOS-2 orthotopic model of osteosarcoma gives rise to large and rapidly growing tumours. By delaying treatment until tumours were palpable, it is possible that tumour energy requirements exceeded capacity of the vascular supply, leading to spontaneous necrosis and apoptosis of the tumour core. Such areas of necrosis and potential apoptosis are commonly observed in clinical tumours at our centre (personal observation).

Ek et al. [7] showed that both StVOrth-2 and StVOrth-3 dramatically restricted spontaneous pulmonary metastases, despite exhibiting differential effects on cell proliferation and collagen I invasion *in vitro*. The authors hypothesized that distinct antitumour pathways may explain these results. With this is mind, the role of these peptides in the metastatic process was again investigated. Pulmonary micrometastases were quantified under low-power microscopy, rather than the postmortem macroscopic method utilised by Ek et al. [7]. Cross-sectional areas of metastatic lesions were quantified to estimate the burden of pulmonary disease. StVOrth-3 showed a dramatic 79% reduction in pulmonary burden of disease, an effect consistent with the observation that StVOrth-3 predominantly restricts adhesion to collagen I [7]. StVOrth-3 may critically inhibit the metastatic cascade via this mechanism; however, one cannot rule out a direct effect on pulmonary lesions via systemic delivery. The method used here for evaluating metastatic lesions is an improvement upon that used by Ek et al. [7].

The final aim of this study was to perform a preliminary evaluation of therapeutic safety. Ek et al. [7] showed that both StVOrth-2 and StVOrth-3 possess a moderate ability to inhibit VEGF expression by SaOS-2 cells. For antiangiogenic agents to be of clinical use, it is critical that these effects are not achieved at the expense of homeostatic processes such as wound healing and inflammation. Serum analysis and histology of harvested viscera did not show any evidence of treatment-related toxicity. All animals appeared to be healthy and exhibited normal grazing and behaviour for the duration of the study. We also did not note any gross differences in surgical wound healing between the treatment groups.

We also clarify that both StVOrth-2 and StVOrth-3 were nonimmunogenic, despite their length (25-mer). There are two direct items that need to be highlighted: (i) both peptides were effective *in vivo* when administered systemically (despite any potential immunogenicity), and (ii) we did not notice any inflammation- or immunostimulation-mediated exudates around the region of the pump outlet. If the peptides were immunogenic, this would be clearly visible in these mice, as we have noticed this before with other anticancer agents (Dass, unpublished data).

StVOrth-2 has been shown to be most effective at inhibiting primary tumour growth while StVOrth-3 predominantly restricts progression of pulmonary metastases. The molecular mechanisms utilised by these peptides are yet to be fully studied and elucidated. Mechanistic studies have focused on the parent PEDF glycoprotein that interacts with regulatory pathways that induce apoptosis of both endothelial and tumour cells [24]. *In vitro* and *in vivo* studies have implicated the Fas-FasL death receptor pathway [25–27], Bcl-2 family proteins [28], caspases, and c-Flip [29]. The current study did not look at molecular markers *in vivo*, apart from the blood analyses for creatinine, alanine transaminase and aspartate transferase. *In vitro* though, we have previously examined several biological and molecular features of PEDF-based peptide treatment of SaOS-2 cells [7]. In that earlier study, StVOrth-2 was the most potent inhibitor of SaOS-2 osteosarcoma cell proliferation, while StVOrth-3 dramatically promoted SaOS-2 adhesion to collagen I. StVOrth-2 and -3 induced osteoblastic differentiation, while StVOrth-3 and StVOrth-4 reduced VEGF expression in SaOS-2 cells. The current set of *in vivo* results, from a focussed pharmacodynamic study, reflect quite closely what we expected from the earlier *in vitro* study.

Peptides have a number of advantages as targeted therapies, being neither genotoxic nor genotype specific [30]. Peptides have excellent tissue penetration and bind with high affinity and specificity to therapeutic targets [31–33]. In this study we demonstrated pronounced differences in action between the two peptides and this begs the question as to precisely which are the aminoacid sequences essential for these effects. StVOrth-2 consisted of $_{78}$LSPLS VATAL SALSL GAEQR TESII$_{102}$, and StVOrth-3 consisted of $_{90}$LSLGA EQRTE SIIHR ALYYD LISSP$_{114}$. The overlapping $_{90}$LSL GAEQR TESII$_{102}$ sequence may in fact be nonessential for the described effects. Further studies utilising the shortened, nonoverlapping sequences of StVOrth-2 and StVOrth-3 are the next logical step. Beyond this, development of a hybrid version of StVOrth-2 and StVOrth-3 peptides may then be possible, leading potentially to a compound that possesses antitumorigenic and antimetastatic properties superior to that of the parent PEDF glycoprotein and hopefully of the individual peptides used in isolation.

Multiple functional epitopes for PEDF have been identified, as well as several distinct PEDF receptors [34, 35]. Filleur at al. [6] first characterised 34-mer (residues 24–57) and 44-mer (residues 58–101) PEDF-derived peptides that conferred antiangiogenic and neurotrophic activity, respectively. Additionally, another sequence, ERT (residues 79–94), showed both antiangiogenic and differentiation activity. Mirochnik et al. [26] derived three different shorter peptides from the 34-mer PEDF-based peptide [6] and demonstrated that a 18-mer peptide was in fact able to inhibit angiogenesis and the growth of prostate cancer in mice. Amaral and Becerra [36] showed that the 34 mer peptide [6] suppresses choroidal neovascularization following subconjunctival administration. The interactions between these various identified epitopes and receptors

are likely to initiate divergent signalling pathways for the different cellular effects of PEDF. We did not identify which PEDF receptor the peptides were interacting with in this study. There is also the possibility that the peptides may be interacting with other cell surface ligands given that the quaternary structure of the peptides is expected to be very different from the full length PEDF protein.

5. Conclusions

This study provides a number of insights into the mechanisms of the antiosteosarcoma activity of PEDF and supports the use of PEDF-derived peptides as therapeutic agents. StVOrth-2 (residues 78–102) primarily restricts growth of primary osteosarcoma, while StVOrth-3 (residues 90–114) restricts pulmonary metastatic disease. These findings are particularly significant given that the orthotopic model of osteosarcoma gives rise to large and rapidly growing tumours, and that these peptides were administered at a stage of advanced disease.

Acknowledgments

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Review Article

Cell and Molecular Biology Underpinning the Effects of PEDF on Cancers in General and Osteosarcoma in Particular

Vijay Chandolu and Crispin R. Dass

School of Biomedical and Health Sciences, Victoria University, Building 6, St Albans, VIC 3021, Australia

Correspondence should be addressed to Crispin R. Dass, crispin.dass@vu.edu.au

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Cancer is becoming an increasingly common disease in which abnormal cells aggressively grow, invade, and metastasize. In this paper, we review the biological functions of PEDF (pigmented epithelium-derived factor) against cancer, with a focus on a particular type of bone cancer called osteosarcoma. PEDF is a 50 kDa glycoprotein and is a potent inhibitor of angiogenesis, via its ability to decrease proliferation and migration of endothelial cells. This paper critically examines the anticancer activities of PEDF via its role in antiangiogenesis, apoptosis-mediated tumor suppression, and increased tumor cell differentiation. Recently, an orthotopic model of osteosarcoma was used to show that treatment with PEDF had the greatest impact on metastases, warranting an evaluation of PEDF efficacy in other types of cancers.

1. Introduction

Cancer is characterized by uncontrolled growth and spread of cells harboring some form of genetic aberration. Formation of tumours requires sequential acquisition of defects that endow tumour cells with the ability to grow, invade, and eventually metastasize. Even after significant improvements in surgery, chemotherapy, and radiotherapy, there are still numerous tumours that are unaffected by therapy. By the time a tumour is diagnosed, it has almost certainly begun metastasizing. In such cases radiation therapy and chemotherapy are often used in combination with surgery in the hope that the tumour and its metastases are effectively controlled [1]. Targeted biological therapies, which are safer, are being studied and clinical trials are conducted to gauge efficacy. The main objective of targeted therapy is to attack specific pathways and tumour growth mechanisms without the side effects attached to small molecule drugs and radiotherapy [2, 3].

PEDF is a 50-kDa glycoprotein, which is a member of the serine protease inhibitor (serpin) family, and it has multifunctional properties [4]. It is found to be a potent inhibitor of angiogenesis, proliferation and migration of endothelial cells, retinal vascular permeability, and tumour activity [5]. PEDF is responsible for the antiangiogenesis in various ocular compartments [6]. These significant antiangiogenic properties led the scientists to shift focus on to studies examining the potential antitumour activities of PEDF. PEDF expression changes in the course of progression of different tumour types [5]. Researchers did a number of studies and showed that there is opposite relation between PEDF levels, grade and metastatic potential of prostate tumours [7], pancreatic adenocarcinoma [8], prostate, melanoma, ovarian, osteosarcoma, glioma [4], hepatocellular carcinoma [9], and Wilm's tumours [10].

The human PEDF gene has been mapped, and it encodes a 418 amino acid protein [11]. It has an asymmetrical charge distribution, with a high density of basic residues concentrated on one side and of acidic residues on the opposite side. Negatively charged acidic PEDF binds to collagen, lacks neurotrophic activity, and may confer antiangiogenic properties (Figure 1). Mutational studies have shown that positively charged amino acids and negatively-charged amino acids are responsible for heparin and collagen binding, respectively [12, 13]. There are three phosphorylation sites identified which collectively induce antiangiogenic and neurotrophic activities. A 34-mer peptide and 44-mer peptide are the two major functional epitopes identified (as shown in Figure 2)

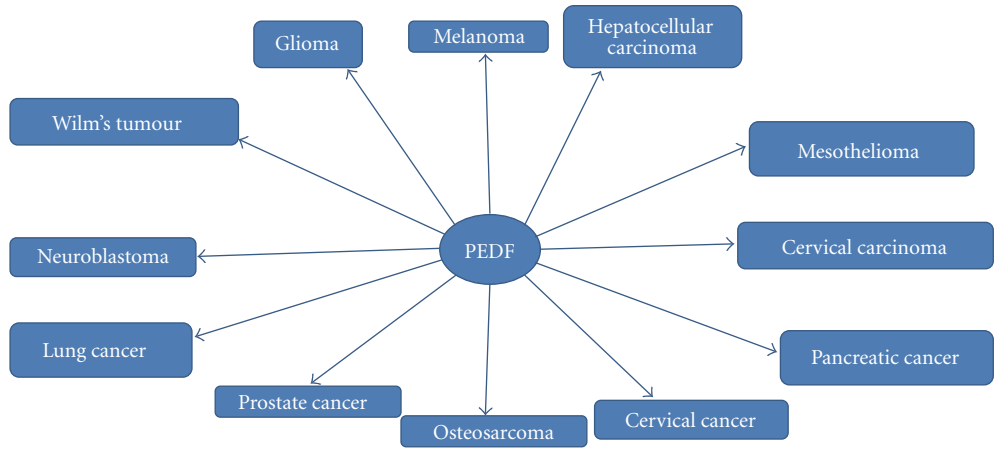

Figure 1: Schematic diagram displaying the functional domains of PEDF. (1) Phosphorylation zones—induce different degrees of antiangiogenic and neurotrophic activity, (2) 34-mer peptide region which has antiangiogenic, blocks cell migration, and induces tumour cell apoptotic activity, (3) 44-mer peptide region which has neurotrophic and cell differentiation activity, (4) basic region (positive) which is necessary for heparin binding, and (5) acidic region (negative) which is responsible for collagen binding and lacks neurotrophic activity.

to date. The 34-mer peptide induces apoptosis, blocks endothelial cell migration and corneal angiogenesis, whereas the 44-mer peptide displays neurotrophic function and the ability to block vascular leakage.

2. Why Use PEDF for Cancer?

PEDF has multiple effects against various types of tumour cells as will be outlined below. Furthermore, PEDF is known to inhibit the proliferation and migration of endothelial cells (ECs) and can also promote apoptosis in ECs [5, 18]. Angiogenesis plays a significant role in cancer progression, promoting growth and metastasis of tumours. Inhibition of the signals that assist new blood vessel growth in tumour(s) has become an important target in cancer therapy [2, 3]. The regulation of angiogenesis involves an inverse relationship between proangiogenic factors and antiangiogenic factors [19]. PEDF not only reduces angiogenesis, but also can increase tumour cell apoptosis and differentiation [5, 18, 20]. These separate functions will be examined in more detail in turn following the next few introductory sections.

3. Cancer Biology—Initiation, Maintenance, and Progression

Cancer is the second biggest cause of death in the USA, next to heart disease [1]. In individuals aged 55 years or above, 78% of all the cancers are being diagnosed. These cancers involve malfunction of genes which control cell growth and division, and only 5% of all the cancers are hereditary. Approximately 11.4 million Americans with a history of cancer were alive in January 2006, according to The National Cancer Institute. In 2010, about 1,529,560 new cancer cases were predicted to be diagnosed, out of which 789,620 are males and 739,940 are females. More than 1500 people per day were expected to die in 2010.

Cancer is a clonal disease which can initiate from a single cell harboring several genetic mutations. Until recently, very little importance was given to the nature of the cell in which the initiating mutation occurred in human cancer [21]. In recent years, it has emerged that perhaps stem cells are the cells of origin for several types of cancer and studies suggest that a stem cell constitutes the target cell in an increasing number of human solid tumours. In favour of the above notion is the finding that not only some leukaemias but also epithelial tumours can originate in normal stem cells (NSCs), and that carcinomas arise after the accumulation of multiple oncogenic events acquired after long periods of time [21].

The majority of cancers occur as a result of alterations in oncogenes. Among these, gene mutation, amplification, or overexpression of HER2/Neu (ErbB2) and epidermal growth factor receptor (EGFR, ErbB1) have been found in various human cancers [22]. Oncogenes encode proteins that control cell proliferation, apoptosis, or both. They can be activated by structural genetic alterations resulting from mutation or gene fusion. For instance, studies of cytogenetics and allelotyping on fresh tumours and cancer cell lines revealed that allelic losses, genetic mutations, and deletions have been found in the 3p21.3 region, which suggests that one or more putative genes (tumour suppressor genes) were being mutated and leading to various types of cancer [23]. Initiation of most types of cancer is mainly due to the functional loss of a tumour-suppressor gene, followed by alterations in oncogenes and additional tumour-suppressor genes [24]. This, in essence, forms the basis of the multihit hypothesis of cancer initiation.

4. Tumour Angiogenesis

The mechanism which involves the growth of new blood vessels from the preexisting blood vessels is called angiogenesis. Via the action of protein factors produced from a tumour in a hypoxic environment, endothelial cells of preexisting

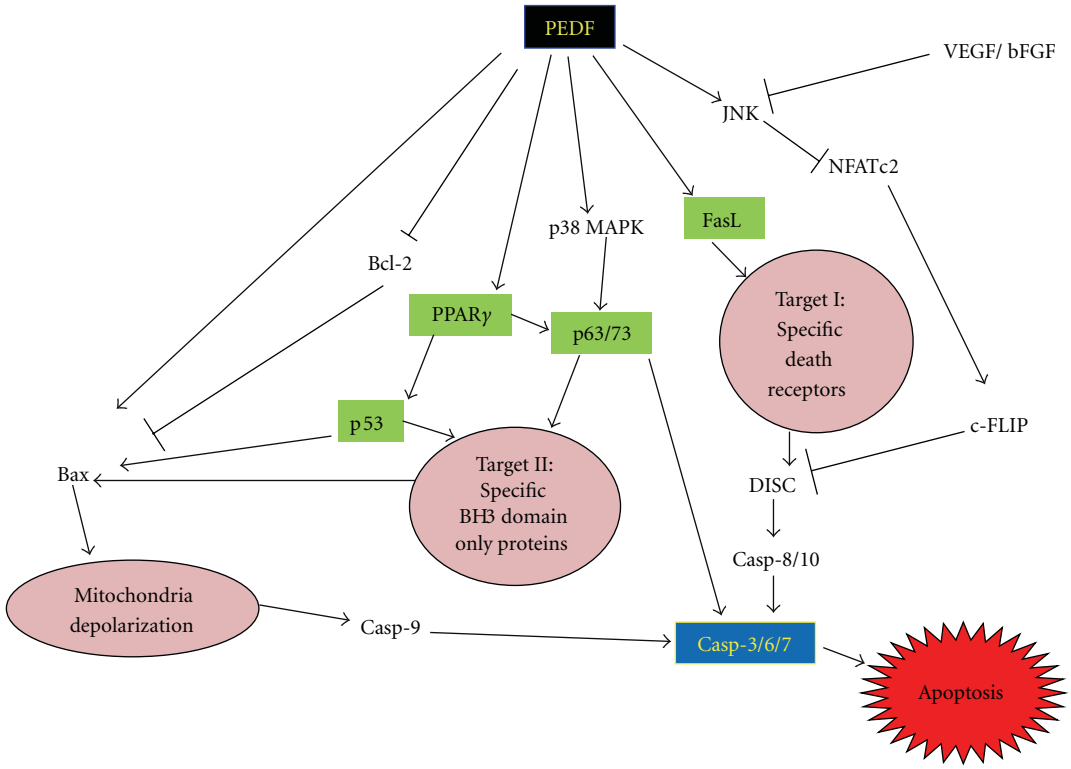

Figure 2: PEDF apoptotic pathways. This schematic chart of apoptotic pathways represents concise information from various sources [5, 14–17]. PEDF apoptotic pathways. PEDF has been noted to induce apoptosis in mammalian cells via these pathways. It can directly stimulate Bax activity, inhibit Bcl-2, signal through PPARγ to activate p53, signal through p38, FasL, and finally through JNK to block c-FLIP. All these pathways culminate in apoptosis.

capillaries acquire a tip cell phenotype [25]. Movement of endothelial tip cells is directed towards incremental levels of growth factors such as VEGF (vascular endothelial growth factor), which has a triple-pronged role:

(1) triggering the permeability of the capillaries and activation of the tip cell phenotype,

(2) promoting migration of tip cells, and

(3) promoting the proliferation and survival of the stalk ECs.

Migration of tip cells is accompanied by the production of extracellular MMPs (matrix metalloproteinases) which are responsible for the remodeling of the nearby ECM (extracellular matrix). It affects the affinity of VEGF species at different extracellular locations. VEGFs have variety of isoforms which are attached to negatively charged molecules in the ECM, and MMPs somewhat control the balance of these VEGF species. For the creation of a functional capillary network and for the determination of specific vascular patterns, the presence of isoforms with diverse capabilities is essential. After generation of a path by the tip cells in the ECM, in order to form a new lumen for blood circulation, a reorientation of the proliferating ECs is required, in precise coordination with pericytes and other stromal components [25]. Further processes such as anastomosis (linkage of different branches on the network), the action of pressure forces and the intrinsic mechanical properties of the tissue, contribute to the formation of the new vessel network and are finely tuned to determine vascular patterning [26]. Hypoxia-inducible factor-1 (HIF-1) production leads to increased VEGF transcription, which causes increased vessel permeability and EC migration and proliferation.

Apart from VEGF, there are many other growth factors responsible for angiogenesis, but VEGF is the most important of all the factors. Hypoxia occurs as the tumour outgrows its existing vascular supply [27]. HIF-1 (hypoxia-inducible factor 1) production only leads to increased production of other proangiogenic molecules such as platelet-derived growth factor (PDGF), transforming growth factors (TGFs) alpha and beta, basic fibroblast growth factor (bFGF), angiotensin, ephrins, thrombospondin-1 (TSP-1), and a class of protein growth factors called angiopoietins (Ang) [28, 29]. VEGF administration can initiate vessel formation in adult animals, but by itself promotes formation of only leaky, immature and unstable vessels. In contrast, Ang1 administration seemingly further stabilises and protects the adult vasculature, making it resistant to the damage and leak induced by VEGF or inflammatory challenges.

bFGF, a member of the fibroblast growth factor family plays diverse roles during embryonic development in regulation of cell proliferation, migration [30], and differentiation.

Angiopoietin-1(Ang-1) [31] plays a significant role at a later stage of blood vessel formation. PDGF plays an important role in normal tissue growth and the pathophysiological processes of vascular diseases like atherosclerosis and restenosis [32]. During the initiation and progression of atherosclerosis, VSMCs are activated by growth factors like PDGF or cytokines, then proliferate and migrate from the media into the intimal surface of the vessel, thus facilitating neointimal formation.

TSP-1 is the first naturally occurring angiogenic inhibitor, which is a multifunctional ECM protein. Downregulation of TSP-1 causes tumour growth alteration by modulating angiogenesis in various types of tumours. It plays a critical role in inhibiting angiogenesis, resulting in the suppression of tumour growth and experimental metastasis [33]. The TGF-β pathway occupies a central position in the signaling networks that control growth and differentiation. TGF-β is known to have pleiotropic effects which differ according to cell state and differentiation. This includes regulation of proliferation and apoptosis, and stimulation of epithelial-mesenchymal transition (EMT) which together is critical for the development of invasive and metastasis potential [34].

Angiostatin (38 KDa), an internal fragment of plasminogen, is a specific inhibitor of EC proliferation. Akin to angiostatin, researchers isolated an angiogenesis inhibitor named endostatin, a 20 kDa carboxyl-terminal fragment of collagen XVIII. It is a specific inhibitor for endothelial cell proliferation and with systemic therapy, causes suppression of tumour-induced angiogenesis and inhibits tumour growth [35]. It has been shown that when a combination of angiostatin and endostatin gene-encoded proteins are delivered to tumour cells by gene transfer, they can exhibit synergistic antiangiogenic and antitumour effects [36].

5. Cell Cycling and Apoptosis in Cancer

The cell cycle plays a significant role in managing to maintain multicellular organisms healthy and alive, in which the parent cell genome undergoes replication and the cell physically divides into daughter cell via cytokinesis. However, genomic stability and cancer may occur due to unwanted mutations caused by some environmental and physiological events. Cells have evolved certain checkpoints to prevent these types of mutations to daughter cells and allow DNA repair mechanism. These checkpoints are controlled by ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related protein) kinases which activate downstream effector kinases Chk1 (checkpoint kinase 1) and Chk2, respectively. Checkpoints will repair damages in most cases, however if the repair mechanisms cannot repair the DNA damage, cells are either permanently withdrawn from the cell cycle or are eliminated by programmed cell death (apoptosis). Cells may reenter the cell cycle in a process called checkpoint recovery [37]. It is known that programmed cell death, that is, apoptosis, is required for normal development of multicellular organisms, whereby unwanted cells are eliminated during physiological and certain pathological conditions.

Apoptosis is a programmed cell death event which occurs during embryogenesis, metamorphosis, endocrine-dependent tissue atrophy, and normal tissue turnover. In multicellular organisms, it is responsible for development, tissue homeostasis, and the immune response via two pathways [38]. They are (i) an intrinsic pathway, which is initiated when the cell is severely damaged, and (ii) the extrinsic pathway, activated when extracellular death ligands are bound by their cognate membrane-associated death receptors.

However, if there is a fault in apoptosis it leads to number of serious diseases including cancer, autoimmunity, and neurodegeneration [39]. When genetically unstable cancer cells tend to acclimatise to a tissue microenvironment that is distant from the primary tumour, a process called metastasis occurs [40]. Metastasis requires the cancer cells to detach from the primary tumour mass, move to and invade blood vessels, survive within the circulation, attach to the endothelium of distant organs, penetrate the endothelial barrier, and establish new tumour colonies. It is this multistage process which is the primary cause of cancer-related deaths, with the primary tumour often resectable. PEDF is responsible for apoptosis of ECs either through the p38/MAPK pathway [14] or through the Fas/FasL pathway [41]. (Detailed PEDF-mediated apoptotic pathways are provided in Figure 2).

6. PEDF and Antiangiogenesis

Angiogenesis is the process by which new blood vessels are formed from a preexisting microvascular network, and it plays a major role in tumour growth and metastasis. For evaluating antitumour efficacy, Yang et al. [42] constructed PEDF expressing-adenovirus (Ad-PEDF), and with successful transfer of the PEDF gene, antitumour efficacy in a mouse tumour model was tested. The tumour volume was inhibited ($2195.1 \pm 462.9\,mm^3$ approximately 50%) with Ad-PEDF when compared to the controls. The mouse which was inoculated with Ad-PEDF showed a 50% survival rate at day 38, than controls (50% on day 13). By using TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, it was determined that the apoptosis index in tumour tissue was higher ($26.3\% \pm 3.3\%$) in the Ad-PEDF group than control groups ($6.3\% \pm 4.7\%$ and $5.6\% \pm 1.9\%$), confirming that increased apoptosis caused the accompanying decreased tumour volume. For elucidating whether the increase in apoptosis of Ad-PEDF-treated tumour tissue was associated with the antiangiogenic effect of PEDF, scientists analysed microvessel density (MVD) of tumour tissues and found that Ad-PEDF demonstrated significant decrease in MVD when compared to control groups.

In [43] determined the growth characteristics of a human malignant melanoma cell line overexpressing human PEDF *in vitro* and *in vivo*. In contrast to the empty vector-transfected group, PEDF overexpression completely inhibited the growth of the tumour. TUNEL assay was performed to determine the extent of apoptosis in the PEDF-transfected

and control vector-transfected tumours and it was found that apoptotic cells increased 30% in the tumours derived from the PEDF-overexpressing G361 cells, when compared with control vector-transfected tumour cells.

In this study, Garcia et al. [44] determined the results of overexpression of PEDF on both melanoma primary tumour growth and metastasis development. Overexpression by melanoma cells inhibited subcutaneous tumour formation. After tail vein injection, PEDF-overexpressing human melanoma cells were unable to grow and metastasise to the lung and liver. The authors also determined the effect of PEDF on proliferation and apoptosis in the subcutaneous tumours and found tumour growth collapse, likely due to impaired vascularisation. PEDF-positive melanoma cells showed significantly lower chemotaxis in a modified Boyden chamber assay. Quantitative data analysis showed a 3- to 5-fold reduction in migration and invasiveness of PEDF positive cells.

A metabolite isolated from ginseng protopanaxadiol saponins called compound K (CK; 20-O-beta-D-glucopyranosyl-20(S)-protopanaxadiol) has an ability to induce apoptosis in various types of cancers. Based on this, Jeong et al. [45] evaluated the antiangiogenic effects of CK *in vitro* and *in vivo*. bFGF induces angiogenesis in human umbilical vein ECs (HUVECs). *In vitro* tests determined that CK inhibited bFGF-induced proliferation, migration and tube formation. *In vivo* antiangiogenic activity of CK was evaluated by a Matrigel plug assay and it was found that CK inhibited bFGF-induced angiogenesis. By measuring the levels of VEGF and PEDF, Jeong et al. evaluated the effect of CK on the phosphorylation of p38/MAPK, and AKT in bFGF-treated HUVECs and found decreased levels of VEGF and increased levels of PEDF following CK treatment. These findings suggest that there is a relationship between PEDF, VEGF, and bFGF with respect to angiogenesis.

7. PEDF and Tumour Cell Differentiation

Angiogenesis underlies the processes of bone growth, repair, and remodelling and may account at least in part for the aggressive nature of osteosarcoma [46]. PEDF regulates angiogenesis to underlie the physiological processes of bone formation, growth, and remodelling [47]. An elegant balance exists between proangiogenic and antiangiogenic factors in bone so that the degree of vascularisation is appropriate for the biology required. VEGF is widely regarded as the most important proangiogenic factor, while PEDF has been identified as the most-potent antiangiogenic factor [18]. The balance between these two factors is as important in bone physiology as it is in other tissues.

PEDF was found to be expressed in the zones of active bone formation and its role in cell differentiation as well as the maintenance of high growth rates in the mesenchymal cell layer was determined by Lord et al. [48] using a deer antler model. Tombran-Tink and Barnstable hypothesized about the significance of PEDF in bone based on RT-PCR and western blot analysis [14], and more recent studies [49] demonstrated that PEDF inhibits osteoclast formation, survival and bone resorption function, and effects were abrogated by VEGF.

PEDF expression by osteoblasts and osteoclasts in regions of active bone formation regulates neovascularisation at these sites, a process that underlies bone formation, growth and remodelling. Without a supporting vasculature, bone precursors are unable to localise to those sites intended for bone development [50]. Angiogenesis in the ossification and lower hypertrophic zones is necessary for the continued growth and functioning of osteoblasts and osteoclasts, enabling the replacement of cartilaginous tissue by bone, a process ultimately regulated by a balance between PEDF and VEGF [47]. Using immunohistochemistry and *in situ* hybridisation, PEDF expression was shown to be largely restricted to the chondrocytes of the avascular resting, proliferative and upper hypertrophic layers of the epiphysis, and to regions of active bone remodelling.

In vitro and *in vivo* studies have revealed that in the case of osteosarcoma, PEDF can induce both indirect and direct suppression of tumour growth and progression by potent antiangiogenic capability of PEDF targeting tumour vasculature and induction of osteosarcoma cell apoptosis, differentiation, and inhibition of cell cycling, respectively [2, 3]. Osteosarcoma usually arises in the metaphysis of long bones. The epiphyseal plate is found between the epiphysis and metaphysis of long bones, which is a natural barrier to tumour invasion. The resistance of epiphyseal cartilage to osteosarcoma invasion is likely to be due to the differential expression of PEDF and VEGF in the zones of the epiphysis [51].

Metastasis is the foremost problem in the treatment of osteosarcoma. *In vitro* studies with rPEDF revealed dose-dependent reduction in cellular invasion by matrigel assay as well as an increase in cell adhesion to collagen type-1 using the human SaOS-2 cell line [52]. *In vivo* studies done on an orthotopic model [53] showed that treatment with PEDF had the greatest impact on metastases. There was a 70% reduction in the development of pulmonary metastases and a 40% reduction in primary tumour size in those mice treated with PEDF [52]. More recently, sustained administration of PEDF via osmotic pumps implanted in mice bearing SaOS-2 tumours demonstrated reduced metastasis to the lungs [54]. Notably, treatment with PEDF was delayed until after the macroscopic appearance of primary tumours. Thus, in this study, both the model used and the delivery regimen were close to the appropriate clinical treatment foreseeable in the future if PEDF is to be used as an osteosarcoma therapeutic.

The critical role of PEDF in regulating MMP activity makes the area of metastasis an intriguing one. Initially, it was found that PEDF can significantly downregulate the levels of MMP-14 but not MMP-2 and -9 in a human chondrosarcoma cell line [55]. This was corroborated by a decreased invasion of cells through collagen-1 matrix in the presence of PEDF. Human melanoma cells were partially inhibited in their invasion, with reduced trafficking of membrane-bound MMP-14 to the cell surface [56].

In [57] demonstrated the ability of PEDF as a multifunctional antitumour agent in neuroblastomas by inhibiting tumour angiogenesis and differentiating tumour cells to

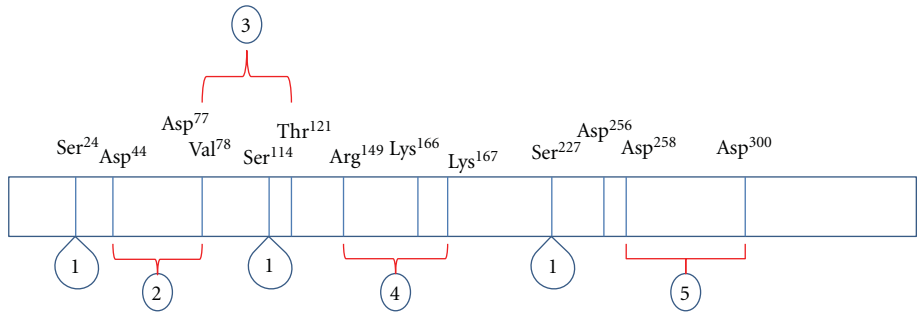

Figure 3: Types of cancers that PEDF has activity against.

produce PEDF. rPEDF cells were injected into the neuroblastoma tumours *in vivo*. After histological examination, PEDF-treated tumour samples resulted in pale regions corresponding to confluent areas of spindle-shaped cells which are characterised by bland nuclei with abundant cytoplasm. These cells were relatively different from the more primitive neuroblasts observed within the tumour distant from the injection site. The control treatments were composed of undifferentiated neuroblast cells. The authors concluded that even a reduced treatment regimen with PEDF *in vivo* was capable to promote discrete areas of tumour cell differentiation.

8. PEDF and Direct Tumour Cell Inhibition

Most of the human ovarian cancers are derived from the ovarian surface epithelium (OSE), which is made of simple squamous to cuboidal mesothelial cells. In one study, Cheung et al. [58] demonstrated the role of PEDF in tumorigenesis of ovarian cancer *in vitro*. MTT cell viability and proliferation assay were performed to determine the physiological functions of PEDF. The treatment with recombinant PEDF (rPEDF) resulted in a dose-dependent decrease in cell growth and concomitant increase in apoptosis, when compared with controls. The role of endogenous PEDF in the regulation of cell proliferation and viability was determined by RNA interference. This study is the first to explain the role of PEDF in OSE biology and ovarian cancer and suggested that the loss of PEDF may be of relevance in carcinogenesis.

In another study, human pancreatic adenocarcinoma cell lines were stably transfected with PEDF [59], and the effects of overexpression investigated in a murine subcutaneous tumour model. Overexpression with a lentivirus-PEDF (LV-PEDF) vector resulted in 28 and 61% inhibition of proliferation and migration of cells, respectively. PEDF overexpression was evaluated *in vivo* and treatment significantly suppressed tumour growth and peritoneal metastasis.

PEDF can suppress osteosarcoma growth, angiogenesis, and metastasis by its multitargeted antitumour activities both *in vitro* and *in vivo* [51]. The *in vitro* anti-proliferative effect was evaluated on two osteosarcoma cell lines rat UMR 106-01 and human SaOS-2 and resulted in dose-dependent reduction in cell proliferation. Varying concentrations of rPEDF were used to determine whether rPEDF induced apoptosis in osteosarcoma, and then analysed by TUNEL assay to determine the percentage of cells undergoing apoptosis and found that rPEDF significantly increased apoptosis and suppressed proliferation of cells. An orthotopic SaOS-2 tumour model [53], consisting of cells injected into the proximal tibiae of nude mice and co-administered with rPEDF showed a significant reduction in primary tumour size, growth rates and a significant reduction in pulmonary metastases.

Osteosarcoma, a prominent primary bone tumour, represents the second highest cause of cancer-related death in childhood and adolescence. Ek et al. [52] demonstrated the potential of plasmid mediated gene transfer of PEDF for direct inhibition of tumour growth, angiogenesis and metastasis in two clinically relevant orthotopic models of osteosarcoma (rat UMR 106-01 and human SaOS-2) via both *in vitro* and *in vivo*. The team examined whether PEDF overexpression influences *in vivo* primary tumour growth and the development of pulmonary metastasis. UMR_{PEDF} (PEDF overexpression) tumours demonstrated slower growth kinetics compared to the parental and vector groups; with a two- to threefold reduction in tumour growth and $SaOS_{PEDF}$ tumours were 59% smaller, respectively. While all mice in the control groups developed lung metastases, only 20% of mice with UMR_{PEDF} tumours and no mice with $SaOS_{PEDF}$ tumours developed pulmonary metastases. Immunohistochemical staining of the tumour microvascular ECs with an antibody against CD34 evaluated that a significant reduction in MVD (microvessel density) in both PEDF-overexpressing tumour groups with an 85 and 74% decrease seen in the UMR_{PEDF} and $SaOS_{PEDF}$ groups, respectively.

PEDF is known as a multifunctional protein which possesses potent antiangiogenic, neurotrophic, neuroprotective, immunosuppressive, and antitumorigenic properties (see Figure 3). Ek et al. [60] identified potential functional epitopes on the PEDF protein sequence and determined their antitumour activity in the human osteosarcoma cell line SaOS-2. They characterized the bioactivity of four synthetic peptides corresponding to sequences 40–64 (StVOrth-1), 78–102 (StVOrth-2), 90–114 (StVOrth-3), and 387–411 (StVOrth-4) of human PEDF using SaOS-2. They evaluated the antiproliferative effects of the peptides and found that StVOrth-2 exhibited the most significant anti-proliferative activity, with a reduction of 59% and 63% seen at day 5 with lower (5 nM) and higher (25 nM) concentrations, respectively. They examined the potential behind suppression of

tumour cell invasion through Matrigel and found that all four peptides significantly decreased Matrigel invasion by greater that 50%. However StVOrth-4 provided that most consistent inhibition, with greater than 70% reduction observed at all concentrations. Ek et al. examined treatment with the PEDF-derived peptides for understanding the alteration of osteosarcoma cell adhesion to collagen (a potential mechanism in antimetastasis) and found that all four peptides considerably increased cell adhesion to collagen type 1, with StVOrth-3 demonstrating 53% greater ability than the other peptides. Prodifferentiation potential studies of the PEDF fragments *in vitro* revealed that administration of StVOrth-1, -2, and -3 significantly increased nodule formation in SaOS-2 cells. They further tested the *in vivo* activity of StVOrth-2 and StVOrth-3 in an orthotopic model of human osteosarcoma, using a model employing tumour cells premixed with peptides, then injected orthotopically. StVOrth-2 exhibited predominantly antiproliferation activity, while StVOrth-3 caused mainly increased collagen adhesion and VEGF suppression. The results of an efficacy study using sustained delivery of peptides in the orthotopic model of osteosarcoma is included in this issue [5, 54].

In a more recent study, Mirochnik et al. [61] have demonstrated improved antiangiogenic activity by the small PEDF peptide, only 18mer in length, called P18. P18 inhibited the growth of prostate tumour growth and the more aggressive Renca cells in vivo in the subcutaneous xenograft model at 10 mg/kg. At the same dose, the 34-mer had no significant effect on tumour growth. The authors stated that P18 was more potent at blocking tumor angiogenesis and causing higher levels of intratumoral apoptosis.

9. Future Directions

PEDF is a promising therapeutic agent for various types of cancers. Until now it is known that it is capable of reducing cancer via inhibiting angiogenesis, tumour growth, cell migration and inducing apoptosis and tumour cell differentiation. Only a little is known about the proangiogenic factors like VEGF, bFGF, PDGF, and there is lot to be known about other proangiogenic factors that interact with PEDF. The fact that regions of PEDF have different functional activity may lead to synthesis of small and cheap peptides which can be tested as anticancer drugs in the near future. Recent biochemical studies have provided some insight into PEDF structure and function. However, very few articles have looked at PEDF receptor-mediated signalling in cancer. Some receptors (G-protein-coupled receptor GPR39) can protect cells from death by increasing secretion of PEDF [62]. More work has to be done in this area given that more than two receptors for this protein have been found to date.

10. Summary

Cancer is characterized by uncontrolled growth and spread of abnormal cells. PEDF is known to be a multifunctional protein which possesses potent antiangiogenic, neurotrophic, neuroprotective, immunosuppressive, and antitumorigenic properties. Scientists designed Ad-PEDF for evaluating the antitumour efficacy and demonstrated role of PEDF in apoptosis and angiogenesis. Other group of researchers demonstrated that overexpression of PEDF suppressed tumour growth and angiogenesis and enhanced the rate of apoptosis. PEDF regulation of angiogenesis underlies the physiological processes of bone formation, growth and remodelling. PEDF can induce both indirect and direct suppression of tumour growth and progression by potent antiangiogenic capability. An orthotopic model of osteosarcoma studies showed that treatment with PEDF had the greatest impact on metastases. There is now justification for evaluation of PEDF efficacy in other types of cancers.

Conflict of Interests

The authors declare that there is not conflict of interests in writing this paper.

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Review Article

Role of Pigment Epithelium-Derived Factor in Stem/Progenitor Cell-Associated Neovascularization

Jung-Tung Liu,[1] Yuh-Lien Chen,[2] Wen-Chi Chen,[3,4] Huey-Yi Chen,[3,4] Yi-Wen Lin,[3] Shu-Huei Wang,[2] Kee-Ming Man,[5] Hui-Min Wan,[6] Wei-Hsian Yin,[7] Po-Len Liu,[6] and Yung-Hsiang Chen[3,4]

[1] *Department of Neurosurgery, School of Medicine, Chung-Shan Medical University and Hospital, Taichung 402, Taiwan*
[2] *Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, Taipei 100, Taiwan*
[3] *Graduate Institutes of Integrated Medicine and Acupuncture Science, College of Chinese Medicine, China Medical University, Taichung 404, Taiwan*
[4] *Departments of Urology, Obstetrics and Gynecology, and Medical Research, China Medical University Hospital, Taichung 404, Taiwan*
[5] *Department of Anesthesiology, Tungs' Taichung Metroharbor Hospital, Taichung 433, Taiwan*
[6] *Department of Fragrance and Cosmetic Science, and Department of Respiratory Therapy, College of Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan*
[7] *School of Medicine, Faculty of Medicine, National Yang-Ming University, Taipei 112, Taiwan*

Correspondence should be addressed to Po-Len Liu, kisa@kmu.edu.tw and Yung-Hsiang Chen, d87a03@ym.edu.tw

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Pigment epithelium-derived factor (PEDF) was first identified in retinal pigment epithelium cells. It is an endogenously produced protein that is widely expressed throughout the human body such as in the eyes, liver, heart, and adipose tissue; it exhibits multiple and varied biological activities. PEDF is a multifunctional protein with antiangiogenic, antitumorigenic, antioxidant, anti-inflammatory, antithrombotic, neurotrophic, and neuroprotective properties. More recently, PEDF has been shown to be the most potent inhibitor of stem/progenitor cell-associated neovascularization. Neovascularization is a complex process regulated by a large, interacting network of molecules from stem/progenitor cells. PEDF is also involved in the pathogenesis of angiogenic eye disease, tumor growth, and cardiovascular disease. Novel antiangiogenic agents with tolerable side effects are desired for the treatment of patients with various diseases. Here, we review the value of PEDF as an important endogenous antiangiogenic molecule; we focus on the recently identified role of PEDF as a possible new target molecule to influence stem/progenitor cell-related neovascularization.

1. Introduction

In the 1980s, pigment epithelium-derived factor (PEDF) was identified and isolated from primary human fetal retinal pigment epithelial cells [1]. It is a 50 kDa secreted glycoprotein that is a noninhibitory member of the serpin (serine protease inhibitor) superfamily of proteins; its gene (*SERPINF1*) is located on chromosome 17p13 [2]. PEDF is an endogenously produced protein widely expressed throughout the human body such as in the eye, liver, heart, and adipose tissue, which exhibits multiple and varied biological activities [3, 4]. PEDF was initially identified as a neurotrophic factor that differentiates retinoblastoma cells into nonproliferating neurons [5]. PEDF has also been shown to be neuroprotective for motor neurons [6], hippocampal neurons [7], dopaminergic midbrain neurons [8], and striatal neurons [9] in different toxin-induced models of neurodegeneration. These effects are parallel to the effects of PEDF on neurogenesis in the context of the neurovasculature. Thereafter, it was further discovered that PEDF also had potent antiangiogenic activity, greater than any other known endogenous factor [10, 11]. The implications of this discovery have proven to be extensive, and many studies have investigated the role of PEDF in various pathological conditions, including chronic

inflammatory disease [12], cardiovascular disease [13, 14], angiogenic eye disease [15], diabetic complications [16], and cancer [17–20].

Although most research has been done in ocular neovascular and neurodegenerative diseases, over the past few years, PEDF has been described as a multifaceted protein with antiangiogenic, antiatherosclerosis, antitumorigenic, antioxidant, anti-inflammatory, antithrombotic, neurotrophic, and neuroprotective properties (Figure 1) [4]. Neovascularization is a complex process regulated by a large interacting network of molecules from stem/progenitor cells [21–24]. More recently, PEDF has been shown to be the most potent inhibitor of stem/progenitor cell-mediated neovascularization [25]. Novel antiangiogenic agents with tolerable side effects are desired for the treatment of patients with various diseases [20, 26]. Here, we review the value of PEDF as an important endogenous antiangiogenic molecule; we focus on the recently identified role of PEDF as a possible new target molecule to influence stem/progenitor cell-related neovascularization therapeutically.

2. Stem/Progenitor Cells and Neovascularization

Angiogenesis and vasculogenesis are the major types of postnatal neovascularization. Angiogenesis is the process where new vessels grow from preexisting blood vessels; whereas vasculogenesis is the process of blood vessel formation occurring by *de novo* production of bone marrow (BM) stem/progenitor cell-derived endothelial cells (ECs), which, in turn, form blood capillaries (Figure 2) [27]. Neovascularization is an important process in the functional recovery of pathological conditions, such as wound healing and ischemic diseases. Hypoxia is an important driving force for neovascularization in various ischemic conditions through stimulation of the expression of many cytokines and growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor, insulin-like growth factor, and fibroblast growth factor (FGF), which play critical roles in induction of neovascularization [28]. Other cellular components including monocytes, T cells, neutrophils, and platelets play significant roles in the induction and modulation of neovascularization. Various stem/progenitor cells are also recruited to the ischemic sites and play crucial roles in neovascularization [29]. Preclinical studies have shown that stem/progenitor cells with or without a combination of growth factors induce neovascularization in ischemic tissues in various animal models [30, 31].

Following ischemia, various angiogenic factors and cytokines are upregulated and promote homing of stem/progenitor cells to the site of injury [32]. It has been shown that circulating stem/progenitor cells could be incorporated into the neovasculature within the ischemic tissue and could differentiate into ECs [33, 34]. Stem/progenitor cells can also differentiate into other supporting cells, which deliver growth factors and cytokines to ischemic tissue and promote angiogenesis through paracrine effects [35]. These cells primarily include various leukocytes as well as fibroblasts and pericytes [36–38]. Stem/progenitor cells in peripheral blood have been shown to differentiate into both early endothelial progenitor cells (EPCs), which function through paracrine effects, and late EPCs, which function directly through vasculogenesis [39, 40].

In the context of EPC biology, vasculogenesis includes the *de novo* formation of vessels via *in situ* migration, proliferation, differentiation, and/or incorporation of BM-derived EPCs into the regenerating vasculature [41]. BM-derived EPCs can localize to vascular structures during skeletal and cardiac ischemia [41, 42], wound healing [43], tumor growth [44], and corneal neovascularization [45]. EPCs also produce a variety of proangiogenic cytokines and growth factors, promoting proliferation and migration of preexisting ECs, activation of angiogenesis, and contributing to vascular regeneration and the reestablishment of tissue homeostasis [46]. Therefore, EPCs function via activation and support of vasculogenesis and may also be major players involved in the activation and mediation of angiogenesis [21], the process of new vessel formation, via *in situ* proliferation and migration of preexisting ECs [47]. This paracrine aspect of EPC activity, reflecting its indirect contribution to neovascularization, was confirmed by several reports demonstrating the presence of various cytokines and other secreting proangiogenic factors in EPCs [48, 49].

3. Ocular Biology

Most diseases cause blindness due to neovascularization. Neovascularization is a complex process regulated in adult tissues by a large interacting network of molecules. Hemorrhaging vessels cause edema and damage to surrounding tissues, particularly the retina. Microvascular lesions often cause severe retinal detachment and loss of vision [15]. PEDF was shown to prevent retinal cell death and counter the abnormal vessel growth induced by VEGF in the eye. Therefore, PEDF has been shown to be the most potent inhibitor of angiogenesis in the mammalian eye and is involved in the pathogenesis of angiogenic eye disease such as proliferative diabetic retinopathy.

The retina is subject to degenerative conditions leading to blindness. Although retinal regeneration is possible in lower vertebrates, it does not occur in the adult mammalian retina. Arnhold et al. [50] determined the potential of adenovirally transduced bone marrow stromal cells (BMSCs) to differentiate into retinal-pigment-epithelial- (RPE-) like cells and evaluated possible rescue effects after transplantation into the retinas of rats [51, 52]. Through an adenoviral vector expressing PEDF, BMSCs were transduced before subretinal transplantation into rats. They showed, for the first time, that BMSCs have the ability to adopt an RPE-like morphology after subretinal grafting into rats. Furthermore, BMSCs were able to induce significant rescue effects for the preservation of photoreceptor cell nuclei. These rescue effects could be increased in dystrophic rats with an adenoviral vector carrying the PEDF gene. Their findings indicate a possible therapeutic option of PEDF for the treatment of

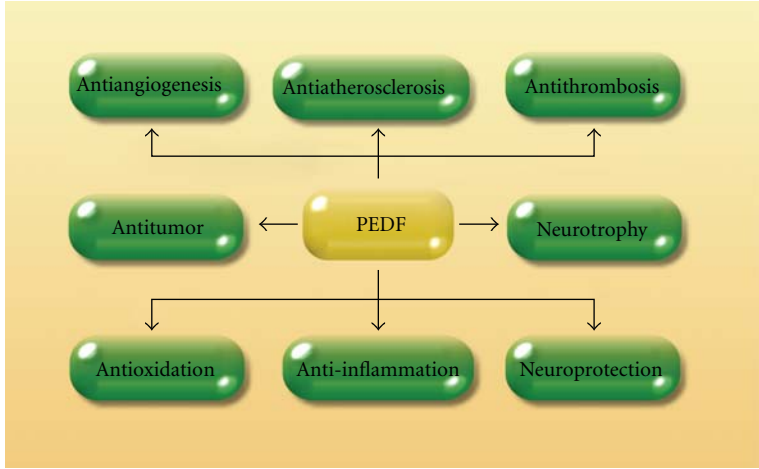

Figure 1: The medicinal properties (antiangiogenic, antiatherosclerosis, antitumorigenic, antioxidant, anti-inflammatory, antithrombotic, neurotrophic, and neuroprotective properties) of PEDF.

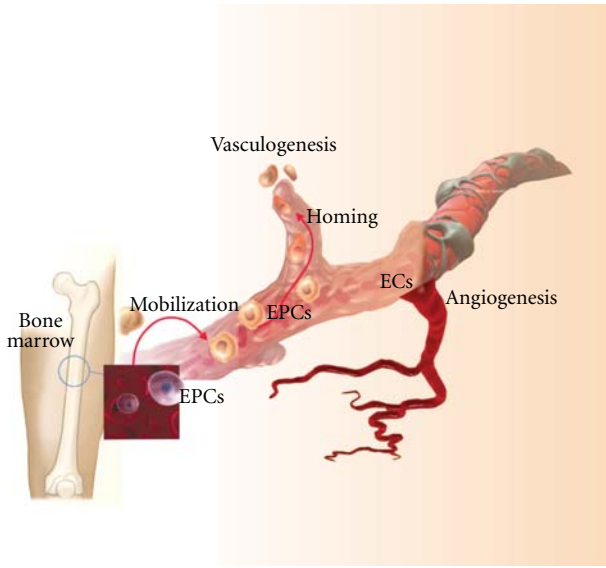

Figure 2: Schematic representation of postnatal neovascularization (angiogenesis and vasculogenesis).

photoreceptor cells and visual loss originally caused by degeneration of the RPE layer [50].

Choroidal neovascularization is a common cause of severe and irreversible visual loss; however, the treatment of choroidal neovascularization has been hindered by its complex and poorly understood pathogenesis [53]. BM-derived cells are postulated to contribute to choroidal neovascularization [54], but little is known about their therapeutic potential for the treatment. Hou et al. reported that BM-derived mesenchymal stem cells (MSCs) transplanted by intravenous injection into a laser-induced mouse model of choroidal neovascularization were specifically recruited into the lesions, where they differentiated into multiple cell types and participated in the process of neovascularization. Engineered MSCs with PEDF at the choroidal neovascularization site inhibited the growth of choroidal neovascularization and stimulated regressive features. Their results suggest that MSCs contribute to choroidal neovascularization and could serve as delivery vehicles of antiangiogenic PEDF for the treatment of a range of choroidal neovascularization-associated eye diseases [55].

Retinal stem cell (RSC) research also offers unique opportunities for developing applications for retinal regeneration therapy. The ciliary body of adult mammals represents a source of quiescent RSCs. These neural progenitors have limited self-renewal potential *in vitro*, but this potential can be improved by mitogens. De Marzo et al. tested combinations of PEDF with FGF during RSC growth to evaluate self-renewal and subsequent differentiation into retinal-like neuronal cell types. It was shown that PEDF might be a modulator during cell division promoting the generation of stem/progenitor cells. Thus, PEDF may contribute to the amelioration of RSC expansion, offering a source of alternative therapy in regenerative medicine (Figure 3) [56, 57].

Human embryonic stem-cell-derived RPE transplantation is a promising therapy for atrophic age-related macular degeneration (AMD). However, future therapeutic approaches might entail cotransplantation of embryonic stem-cell-derived RPE with retinal progenitor cells (RPCs) as a replacement source for lost photoreceptors. Zhu et al. determined the effect of polarization of embryonic stem-cell-derived RPE monolayers on their ability to promote survival of RPCs. They found polarized embryonic stem-cell-derived RPE cells secrete high levels of PEDF that can support RPC survival suggesting that polarization of embryonic stem-cell-derived RPE would be an important feature for the promotion of RPC survival in future cell therapy for atrophic AMD [58]. In addition, Vaajasaari et al. reported the differentiation of functional RPE-like cells from several stem cell lines in culture conditions. The differentiated cells were able to secrete PEDF characteristic of native RPE cells. Their results showed that RPE-like cells can be differentiated in xeno-free, defined culture conditions, which is mandatory

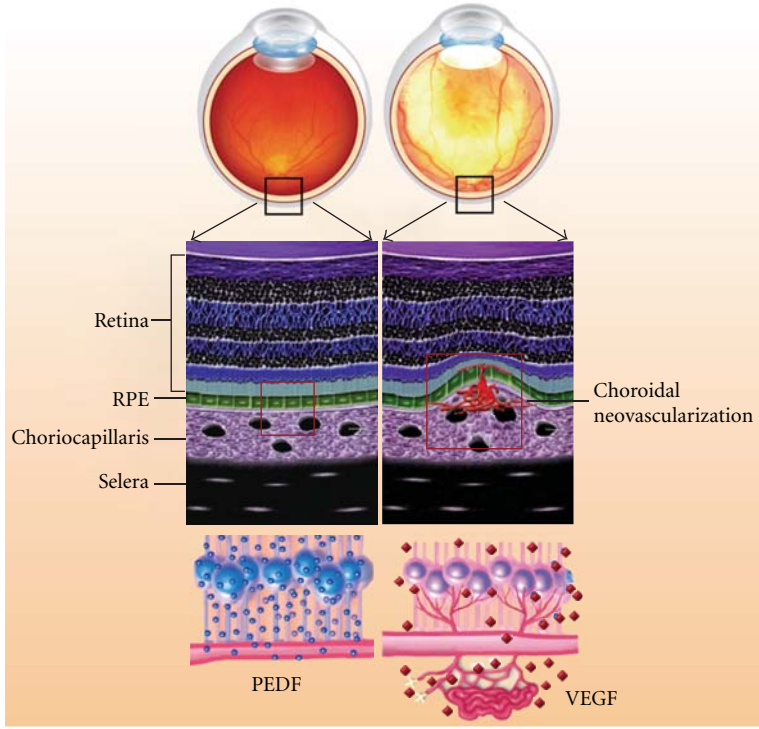

Figure 3: Schematic representation of choroidal neovascularization. PEDF was shown to prevent retinal cell death and counter the abnormal vessel growth induced by VEGF in the eye.

for good manufacturing practice production of these cells for clinical use [59].

Proliferative diabetic retinopathy is characterized by pathological retinal neovascularization. PEDF contains an N-terminal 34-amino acid peptide (PEDF-34). Longeras et al. presented data that PEDF-34 also possesses antivasculogenic activity; PEDF-34 attenuates EPC mobilization from the BM into circulating blood during retinal neovascularization [25]. Since PEDF controls the neuroprotective and antineovascular regulatory axis that determines cell growth, it could be used in combination therapeutic strategies for ocular neovascular diseases [60].

4. Cancer Biology

Cancer remains a major medical problem associated with considerable morbidity and mortality [61]. It is important for researchers to improve the current therapeutic agents for cancer treatment, particularly targeting inhibition of tumor growth, survival, and metastasis. Cancer stem cells (CSCs), a special subpopulation of tumor cells, are considered to be tumor-initiating cells. More recently, these cells have also been identified as initiators of tumor neovascularization [62]. Vasculogenic mimicry—a newly defined pattern of tumor blood supply—provides a special passage without ECs and is conspicuously different from neovascularization. The biological features of the tumor cells that form vasculogenic mimicry remain unclear. CSCs are believed to be tumor-initiating cells, capable of self-renewal and multipotent differentiation, which resemble normal stem cells in phenotype and function. CSCs have recently been shown to contribute to vasculogenic mimicry formation as well as angiogenesis. The importance of vasculogenic mimicry in tumor progression suggests that it could constitute a novel therapeutic target for cancer [63].

Many researchers are investigating the crucial role of the proangiogenic factor VEGF in tumor angiogenesis, where the formation of new blood vessels carrying essential nutrients to the tumor cell becomes a critical factor for tumor growth [64]. Since VEGF plays an integral role in mediating tumor angiogenesis and tumor cell survival, current efforts are dedicated to developing therapeutic agents against VEGF; one emerging candidate is PEDF [65]. PEDF has recently shown promise as a potential antitumor agent, causing both direct and indirect tumor suppression. Here, we briefly introduce the unique antitumor properties of PEDF and discuss its role as an effective antiangiogenic, antiproliferative, and prodifferentiation factor (Figure 4).

The poor outcome of cancer gene therapy in clinical trials relates, in part, to insufficient gene delivery to tumor sites. MSCs represent a new tool for the delivery of therapeutic agents to tumor cells [66, 67]. Fitzgerald et al. used tumor cells overexpressing PEDF to establish PEDF as both a metastatic suppressor and a neuroprotectant in the brain *in vivo* [68]. Gao et al. used a nude mice model of hepatocellular

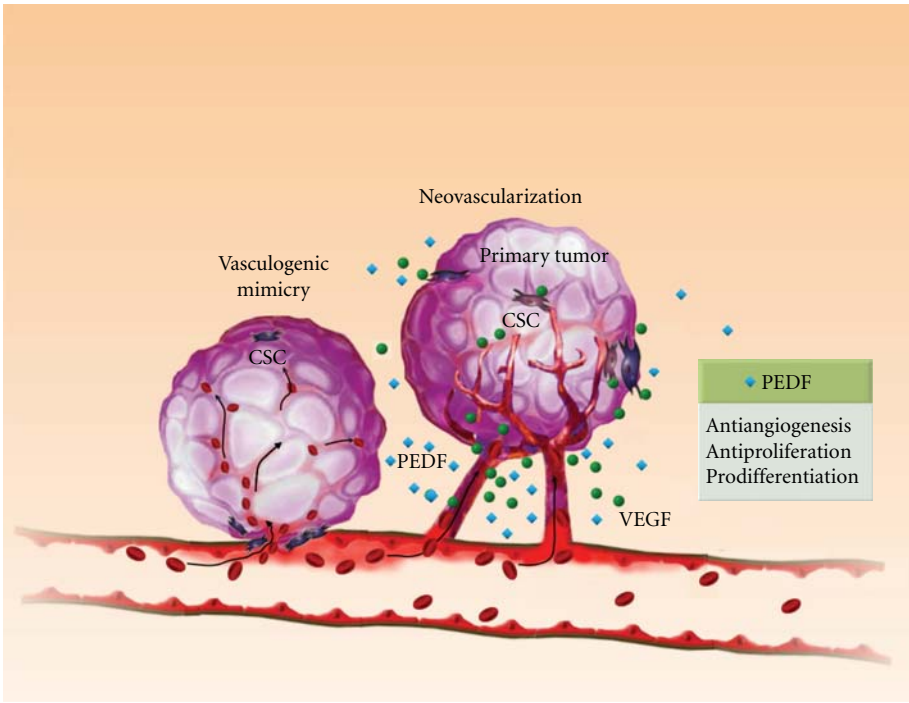

Figure 4: Schematic representation of CSC participation in vasculogenic mimicry and neovascularization initiated by the presence of EPCs. Like in tumor angiogenic sprouting, neovascularization starts with the secretion of proangiogenic factors (such as VEGF) by the tumor under hypoxia. The proangiogenic factors circulate into the bloodstream towards the bone marrow, targeting the release of EPCs. The activated EPCs form a column from the existing blood vessel. PEDF has antitumor properties as an effective antiangiogenic, antiproliferative, and prodifferentiation factor.

carcinoma (HCC) to evaluate the potential of genetically modified human MSCs to function as an effective delivery vehicle for therapeutic genes. MSCs derived from the BM were efficiently engineered to express human PEDF by lentiviral transduction and then tested *in vitro* for high-level expression and bioactivity of the transgenic protein. The preferential homing of MSCs toward HCCs was confirmed by *in vitro* and *in vivo* migration assays. *In vivo* efficacy experiments showed that intravenous injection of PEDF-expressing MSCs significantly suppressed both the growth of primary liver tumors and the development of pulmonary metastases. Moreover, MSC-based PEDF gene delivery moderately increased systemic levels of human PEDF. Immunohistochemistry of primary liver tumors demonstrated lower microvessel density in mice treated with MSC-PEDF than in control mice. This study shows, for the first time, the tropism of MSCs derived from the BM for HCC. MSCs can be genetically modified *ex vivo* to express the PEDF gene that has therapeutic efficacy against HCC. Their results suggest a potential role of MSCs as a targeted, therapeutic delivery vehicle for the treatment of HCC. Although this study indicates that PEDF is a good therapeutic agent worthy of assessment in HCC, the same approach might also be exploited in the treatment of patients with other tumor types [69]. A better understanding of the contribution of PEDF and CSCs to neovascularization should elucidate the mechanisms of cancer initiation and progression as well as establish new concepts for cancer diagnosis and treatment [70, 71].

5. Cardiovascular and Neurovascular Biology

Cardiovascular and neurovascular diseases are worldwide causes of morbidity and mortality [72–76]. Stem cells and EPCs have been studied as novel and promising strategies for the treatment of these vascular-associated diseases [35]. Adult peripheral blood contains BM-derived EPCs with properties and markers that are similar to embryonic-derived angioblasts [77]. Recent studies have shown that BM-derived stem/progenitor cells can repair the endothelium, in contrast to the traditional concept that postnatal tissue revascularization was achieved by neighboring endothelial replication [78]. The progressive impairment of endothelial function and integrity starts a cascade of events leading to microcirculation damage [79], atherosclerosis, and common cardiovascular and neurovascular diseases such as coronary heart disease (CHD), myocardial infarction (MI), heart failure (HF), peripheral arterial disease (PAD), dementia, and stroke [80]. The proliferation rate of ECs is very low in adults, which limits the contribution of ECs to neovascularization. EPCs promote vascular repair and provide the rationale for autologous stem cell therapy [81]. Recently, EPCs have been vigorously investigated in various fields of medicine. Changes in EPC number have also been investigated in diseases other than cardiovascular and neurovascular diseases, such as metabolic disorders, neoplastic pathologies, rheumatic disease, chronic kidney disease, and chronic obstructive pulmonary disease [35]. In this section, we discuss the role

of PEDF as a possible new target molecule to therapeutically influence cardiovascular and neurovascular diseases.

PEDF has been characterized in cardiovascular systems. It has a protective role in atherosclerosis, the main cause of CHD, MI, and HF, due to its anti-inflammatory, antioxidant, and antithrombotic effects in the vessel wall and platelets. Expression of PEDF by ECs is essential for the inhibition of proliferation and migration of smooth muscle cells after balloon injury [82]. The antioxidative properties of PEDF have been shown to block TNF-α-induced EC activation [83]. These observations suggest that PEDF might have beneficial effects on atherosclerosis by suppressing inflammatory proliferative responses to injury. Additionally, PEDF has strong antiangiogenic effects by inducing apoptosis [84] in ECs and by regulating the expression of other angiogenic factors. Local blocking of PEDF (e.g., in ischemic tissue in the heart) might favor angiogenesis, induce neovascularization, and lead to increased perfusion of the injured tissue. In contrast, local overexpression of PEDF restricted to atherosclerotic lesions might block angiogenesis, inflammation, and thrombosis at these sites and thus counteract destabilization and rupturing of the lesion otherwise caused by inflammatory activation [85] and excessive angiogenesis, thereby inhibiting subsequent thrombus formation [4].

In our previous study, we described a streamlined method for the rapid isolation, growth, and *ex vivo* expansion of late outgrowth ECs from Wharton jelly of a human umbilical cord; we evaluated the ability of these cells to reendothelialize and inhibit neointimal hyperplasia in injured femoral arteries of mice. We also examined the direct effects of EPC-conditioned medium on the migration and proliferation of human aortic smooth muscle cells and the role of PEDF in these effects. Our results showed that EPC transplantation led to rapid reendothelialization of denuded arteries, which resulted in significant inhibition of neointimal thickening. This was the first report demonstrating that EPCs derived from an umbilical cord aid in accelerating reendothelialization and attenuating vascular remodeling at sites of arterial injury; these effects were closely associated with PEDF [14]. These findings have implications for a novel PEDF-related and cell-based therapy for cardiovascular and neurovascular diseases.

MSCs can ameliorate MI injury; however, MSCs from older donors are less efficacious than those from younger donors. More recently, Liang et al. determined how age-related expression of PEDF affects MSC therapeutic efficacy for MI. Their data showed that PEDF expression was increased in MSCs from old mice compared to young mice resulting in significantly impaired therapeutic efficacy in old MSCs, compared with that in young MSCs, for treatment of mice subjected to MI. PEDF overexpression in young MSCs impaired the beneficial effects against MI injury and induced cellular profile changes in the infarct region that was similar to administration of old MSCs. Knocking down PEDF expression in old MSCs improved MSC therapeutic efficacy and induced a cellular profile similar to administration of young MSCs. PEDF secreted by MSCs regulated the proliferation and migration of cardiac fibroblasts. These data provide the first evidence that the paracrine factor PEDF plays a critical role in the regulatory effects of MSCs against MI injury. Furthermore, the impaired therapeutic ability of aged MSCs is predominantly caused by increased PEDF secretion [86]. These findings indicate PEDF as a promising novel genetic modification target for improving aged MSC therapeutic efficacy.

Vascular and neural tissues are delicately intertwined in functional neurovascular units. This codependence emerges early in development with the coordinated growth and tissue modeling of both cellular elements [87]. Stem/progenitor cells in the developing central nervous system and in neurogenic regions of the adult brain are stimulated to self-renew and generate more neurons by factors released from various vascular cells [88]. In the mammalian brain, neurogenesis persists in 2 germinal areas, the subventricular zone (SVZ), and the hippocampus, where continuous postnatal neuronal production seems to be supported by neural stem cells (NSCs) [89]. Ramírez-Castillejo et al. identified PEDF as critical for the communication between vascular and neural cells in an adult NSC niche, the SVZ [90]. A single factor that can stimulate brain tumor cells to differentiate and, at the same time, cut off their blood supply has unlimited therapeutic value. PEDF levels decline with aging, possibly contributing to cell senescence and to age-related susceptibility to cancer [91]. These data demonstrate that PEDF is a niche-derived regulator of adult NSCs and provide evidence for a role for PEDF in NSC maintenance.

The potential of NSCs for brain repair depends on their capacity for self-renewal. Recent evidence for the close apposition of adult periventricular NSCs and blood vessels has confirmed the findings [88, 90] that factors derived from the vasculature contribute to regulation of the adult NSC pool [92, 93]. A new study by Andreu-Agullo et al. revealed that the vasculature-derived PEDF promotes the Notch signaling-dependent renewal of adult periventricular NSCs through an unconventional mechanism [94]. They found that Notch was active in astroglia-like NSCs but not in transit-amplifying progenitors of the murine subependymal zone and that the level of Notch transcriptional activity correlated with self-renewal and multipotency. Moreover, dividing NSCs appeared to balance renewal with commitment via controlled segregation of Notch activity. PEDF enhanced Notch-dependent transcription in cells with low Notch signaling, thereby subverting the output of an asymmetrical division to the production of 2 highly self-renewing cells. Mechanistically, PEDF induced a noncanonical activation of the nuclear factor (NF)-κB pathway. These data provide a basis for stemness regulation in vascular niches and indicate that Notch and PEDF cooperate to regulate self-renewal [94].

6. Conclusions

PEDF has been described as a natural angiogenesis inhibitor with neurotrophic and immune-modulation properties [95]. It balances stem/progenitor cell-associated angiogenesis in the eye, as well as cardiovascular and neurovascular systems, and blocks tumor progression. The mechanisms underlying most of these events are not completely clear; however, it

appears that PEDF acts via multiple high affinity ligands and cell receptors. In this paper, we summarized the current knowledge on the important endogenous antiangiogenic molecule PEDF; we focused on the recently identified role of PEDF as a possible new target molecule to influence stem/progenitor cell-related neovascularization. We discussed the multimodal activities of PEDF and addressed the therapeutic potential of PEDF in treating angiogenesis-, neurodegeneration-, and inflammation-related diseases. However, many questions remain to be resolved; these specific points must be addressed prior to initiation of any human clinical trials using PEDF peptides.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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Review Article

The Emerging Role of PEDF in Stem Cell Biology

Mina Elahy, Swati Baindur-Hudson, and Crispin R. Dass

Department of Biomedical and Health Sciences, Victoria University, St Albans, VIC 3021, Australia

Correspondence should be addressed to Crispin R. Dass, crispin.dass@vu.edu.au

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Encoded by a single gene, PEDF is a 50 kDa glycoprotein that is highly conserved and is widely expressed among many tissues. Most secreted PEDF deposits within the extracellular matrix, with cell-type-specific functions. While traditionally PEDF is known as a strong antiangiogenic factor, more recently, as this paper highlights, PEDF has been linked with stem cell biology, and there is now accumulating evidence demonstrating the effects of PEDF in a variety of stem cells, mainly in supporting stem cell survival and maintaining multipotency.

1. Introduction

1.1. Introduction to PEDF. Pigment-epithelium-derived factor (PEDF) is a glycoprotein that belongs to the superfamily of serpin protease inhibitor proteins without inhibitory function, encoded by the gene SERPINF1 located on chromosome 17p13 which is well conserved in evolution [1]. It is a protein of 418 amino acids, with a size of 50 KDa and widely expressed in most body tissues [2]. It is an extracellular protein which shows the secondary and tertiary structure of serpin and binds to collagen-1 and heparin. The $\alpha\beta$-sheet is the dominant feature of the secondary structure and comprises the core structural domain of the protein, being closely involved in dynamic movements that are part of serpin function [3]. Existence of a reactive centre loop (RCL) is another feature of serpins, and it is a proteinase recognition site and a critical component of the function of serpins [4]. PEDF contains an RCL structure but the function of this is still unknown [4].

PEDF was originally isolated from the conditioned medium of cultured human fetal retinal pigment epithelium cells [4]. As mentioned before, it can be found in almost all tissues but the highest amount of expression has been observed in the eye, fetal and adult liver tissue, adult testis, ovaries, placenta, and the pancreas. A significant reduction in the expression of PEDF is found in senescent (aging) cells [5].

PEDF is a pluripotent molecule with neurotrophic qualities, and several biological activities have been ascribed to it, including antiangiogenic, antiproliferative, prodifferentiation, neuroprotective, anti-inflammatory, and antitumour properties [6–8]. The antiangiogenic properties and neurotrophic activities of the protein are due to peptides derived from the N-terminal region of this protein, while the C-terminal peptides interact with its membrane receptor [7]. How PEDF controls all these biological processes and how it intercepts growth-promoting signals, accelerates cell death cascades, and prolongs cellular lifespan remains widely unknown but it presumably binds to cell surface receptors to trigger various signalling cascades. Evaluating the expression of different PEDF receptors (PEDF-Rs) could help to determine the specific biological responses of PEDF. Studies show that there are at least two different PEDF-Rs, specific to neural or endothelial cells, respectively [9]. The signalling pathways activated by PEDF regulate a number of key transcription factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [10], nuclear factor of activated T cells (NFATs) [11], peroxisome proliferator-activated receptor (PPAR-γ) [12], and the potent promigratory urokinase-type plasminogen activator (uPA)/receptor (uPAR) system [8, 13].

1.2. Introduction to Stem Cell Biology. Stem cells are recognised by two important features, firstly the self-renewal property and secondly the multilineage differentiation potential [14]. These characteristics make stem cells a preferred

candidate for cell-based therapy for diseases such as neuronal damage [15].

Generally, stem cells are divided into two groups based on their origins: embryonic stem cells (ESCs) which are harvested from the inner cell mass of blastocysts and have the ability to produce all three embryonic germ layers-ectoderm, endoderm, and mesoderm and adult stem cells which are critical for tissue homeostasis. Adult stem cells support tissue regeneration and replacement of ongoing cell loss due to natural cell death and injury. These cells can be found in most tissues such as the brain, bone marrow, liver, and retina [16, 17].

Stem cells divide via mitosis and produce one daughter cell to replace the divided parent cell (and thereby maintain the stem cell pool) and one other cell which will go through the differentiation process. The cell which undergoes differentiation can do so to different types of cells. Recently, a phenomenon known as trans-differentiation has been identified which is when tissue-specific stem cells switch their lineage to that of some other tissue under suitable conditions. For example, bone marrow stem cells are able to transdifferentiate into skeletal muscle, smooth muscle, and neuronal cells [18].

In spite of the unique properties of stem cells (self-renewal and differentiation), adult stem cells stay dormant through most of their lifetime and are activated under certain circumstances by specific environmental factors [19]. The specific surrounding environment includes stroma which contains fibroblasts, macrophages, neutrophils, endothelial cells and other differentiated cells, and the microenvironment around the stem cell [20].

The surrounding microenvironment of a stem cell is known as the stem cell niche and it includes different signalling areas which helps the daughter cell commit to different fates. Stem cells need to be held within the niche and this happens via adhesion between stem cells and underlying basement membrane or support cells. Upon division, if a cell is placed outside the niche, it commits to differentiation depending on the different microenvironmental stimuli and signalling it encounters in its new niche [19].

Stem cells have the capacity to react to a broad range of growth factors and signalling molecules and express many of the important downstream signal transduction components such as those signal transduction pathways that are present and may be active in stem cells. These include Notch, transforming growth factor beta (TGFβ), Wnt, bone morphogenetic proteins (BMPs), Hedgehog, and Janus kinase/signal transducer and activator of transcription (Jak/Stat) family members [21].

2. PEDF and Stem Cells

2.1. PEDF and Human Embryonic Stem Cells. Human embryonic stem cells (hESCs) grown *in vitro* require feeder cells such as fibroblasts to maintain proliferation and pluripotency. In a study using microarray analysis to study the gene expression profile of human foreskin fibroblasts, a number of molecular targets were identified that are potentially involved in the ability of these cells to act as feeder cells for hESCs. The most likely target genes they found were PEDF, c-Kit, and leptin. These findings were supported by real-time polymerase chain reaction (RT-PCR) and virtual serial analysis of gene expression (SAGE) analysis. It has been hypothesized that the protein products of these genes may play an important role in the support of hESC growth by human foreskin fibroblasts [22].

Age-related macular degeneration (AMD) is one of the common causes of blindness in the elderly and is characterised by degeneration and loss of retinal pigmented epithelial (RPE) cells and photoreceptor cells in the macular region. Local expression of PEDF is significantly decreased in the eyes of patients with AMD [23]. There are two late forms of the disease: in geographic atrophy (also known as dry) there are confluent areas of RPE degeneration and loss in the macular region, while in neovascular AMD (also known as wet), there is growth of blood vessels from the choroid through Bruch's membrane to the subretinal space—a process known as choroidal neovascularization (CNV) [24, 25].

hESC-derived retinal pigment epithelium (hES-RPE) seems to be a promising strategy for cell replacement in this condition. Studies have shown that polarised hES-RPE cells secrete high levels of PEDF and that culture medium containing this PEDF increased proliferation and supported the survival of retinal progenitor cells (RPCs) isolated from human fetal eyes [23]. This role of PEDF was confirmed by the addition of neutralising PEDF antibody to the culture media, which diminished or completely abolished the increase in RPC proliferation and survival.

Apart from the potential effects of PEDF on RPCs, PEDF also has at least three important effects on the health of RPEs. The first effect is the neuroprotective activity towards photoreceptors and other retinal neural cells against damage of cytotoxic injury. Second, its antiangiogenic effect prevents pathologic neovascularisation through inhibiting endothelial cell migration and promoting endothelial apoptosis. Third, its antiaging function inhibits premature senescence of both RPE cells and their neighbouring retinal cells [26] (Table 1).

2.2. PEDF and Mesenchymal Stem Cells (MSCs). Bone marrow-derived stem cells (BMSCs) are also called mesenchymal stem cells (MSCs) because these cells are able to differentiate into a variety of mesodermal tissues including bone, cartilage, and adipose [14]. One of the most abundant proteins identified in murine mesenchymal MSC-conditioned medium is PEDF [27]. Immunofluorescent staining shows a high level of expression of PEDF in the rough endoplasmic reticulum/Golgi areas [27]. PEDF is also found to be located near the plasma membrane and in the extracellular space (considering the ability of PEDF to bind to collagen and proteoglycans in the extracellular matrix).

The role of bone-marrow-derived SCs (BMSCs) or MSCs in angiogenesis is not clear but it has been suggested that they may support and stabilise newly formed blood vessels. The process of angiogenesis is regulated by various factors that stimulate or inhibit angiogenesis. One of the key factors that

Table 1: Effects of PEDF on different types of stem cells.

Stem cell type	PEDF effect	Reference
Human embryonic stem cell	Proliferation, support, and survival	[22, 23]
Neural stem cell	Self-renewal Maintenance of multipotency Activation of cell division Antiapoptotic effects	[31–33]
Retinal stem cell	Self-renewal Cell expansion Inhibits migration of and induces apoptosis of endothelial cells Regression of CNV (at low doses) Development of neovascularisation (at high doses)	[35, 37, 38]

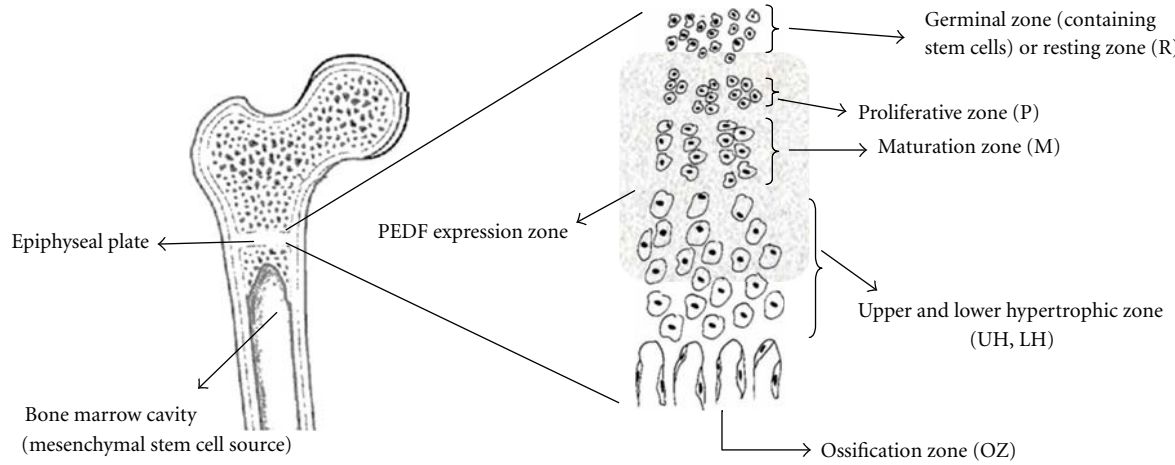

Figure 1

stimulates angiogenesis is vascular endothelial growth factor (VEGF). This factor is expressed in a wide range of cells including BMSCs. On the other hand, one of the key functions of the PEDF molecule is the inhibition of angiogenesis. In a study by Fan et al. (2011), it was observed that PEDF expression was much stronger than that of VEGF in BMSCs. Based on data acquired during that project, the authors concluded that BMSCs may not usually be an angiogenesis-promoting population in a normal environment [28]. This view was supported by other findings that, in the presence of high cell numbers, MSCs are capable of inhibiting capillary growth [29].

During differentiation of MSCs to osteoblasts, expression of several genes begins and this includes PEDF. It has been shown that a high level of PEDF is expressed during early stages of bone development by osteoblasts and to a lesser extent in osteoclasts [30, 31]. Osteoblasts and possibly osteoclasts are able to synthesise and release PEDF, and this protein has a critical role in normal and abnormal bone angiogenesis. In developing bones, blood vessel growth is localised. In locations such as the long bone growth plate, blood vessels selectively invade the region between hypertrophic chondrocytes and newly formed bone matrix (Figure 1). These newly formed vessels allow migration of osteoblasts which leads to new bone matrix deposition and bone elongation. PEDF is expressed in the epiphyseal cartilage and in the areas of active bone remodelling in the primary spongiosa and periosteum of metaphyseal bone. There is a gradual decrease in the intensity of PEDF expression as chondrocytes differentiate toward the base of the growth plate [30, 32].

It has been found that PEDF secreted from enriched mouse bone marrow cells exhibiting surface markers characteristic of multipotent MSCs is able to attract fibroblasts and this property may play a role in regulating the cellular profile at the site of an injury. PEDF secretion happens in early postinjury stages rather than late postinjury and is due to the different cellular requirements during different stages. For example, PEDF released by resident stem cells may stimulate migration of cells needed early in regeneration, such as fibroblasts while inhibiting migration of cells such as endothelial cells needed for capillary formation at subsequent stages of healing [27].

2.3. PEDF and Neural Stem Cells (NSCs). In 2006 Ramírez-Castillejo et al. [33] showed that PEDF produced by the

subventricular zone (SVZ) in the mouse brain promotes self-renewal and invigoration of slowly dividing adult neural stem cells (NSCs) *in vitro* [33]. It also stimulates the expansion of the stem cell niche in brain and induces differentiation toward the neuronal phenotype in multiple cell types [34]. In the subependymal zone (SEZ) of the adult mammalian brain, neuroblasts and oligodendrocytes are produced from NSCs through fast-dividing transit-amplifying progenitors (TAPs). Vascular elements and several endothelium-derived factors are known to regulate the proliferation and/or survival of neural progenitors and PEDF is one of them. PEDF acts as an endogenous SEZ niche factor that can evoke NSC expansionary divisions [35].

Andreu-Agulló et al. (2009) found that PEDF could modulate the balance between symmetric and asymmetric divisions in NSCs. Essentially PEDF promoted self-renewing divisions and maintenance of a multipotent state in NSCs through its effects on Notch transcriptional activity. The mechanism by which this occurs appears to be complex. Activation of Notch receptors by membrane bound ligands results in the generation of an intracellular domain of Notch (NICD) which moves to the nucleus, where it binds the repressor C promoter-binding factor 1 (CBF1). CBF1 can then bind to the promoters and initiate transcription of genes in the *Hes1* and *Herp* families and, possibly, the *Egfr* gene [35]. This results in the effects of Notch signalling, that is, increase in self-renewal of NSCs.

So what is the role of PEDF in all this? Notch activity is attenuated by the interplay between transcriptional co-activators and corepressors. One of these is N-CoR, which is a corepressor for a number of nuclear receptors and can bind to CBF1. It was postulated that the CBF1-binding sites in the *Hes1* and *Egfr* promoters were occupied by N-CoR, repressing the transcription of these genes (Figure 2(a)). It was found that, in the presence of PEDF, N-CoR moves from a nuclear to cytoplasmic location and that PEDF removes N-CoR (the repressor) from the CBF1-binding sites in the promoters of the *Hes1* and *Efgr* genes, thus allowing CBF1 (the inducer) to bind and activate these genes (Figure 2(b)). Further experiments suggested that PEDF carries out these activities through a noncanonical activation of the NF-κB pathway [35].

2.4. Stem Cells Overexpressing the PEDF Gene. MSCs have been shown to differentiate into endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) and incorporate into the new blood vessel wall and form vascular tubes. MSCs have an explicit role in various proangiogenic models although, interestingly, they have an antiangiogenic effect on corneal wound healing after chemical injury [36]. Excessive vascularisation can lead to pathological situations. Studies show that MSCs contribute to the formation of choroidal neovascularisation (CNV) and they can differentiate into both vascular and extravascular cells in CNV [37]. CNV is a major form of ocular angiogenesis which leads to visual loss.

The mechanism of CNV is complex but, basically, a disrupted balance between angiogenic and antiangiogenic factors like VEGF as an angiogenic stimulator and PEDF as an

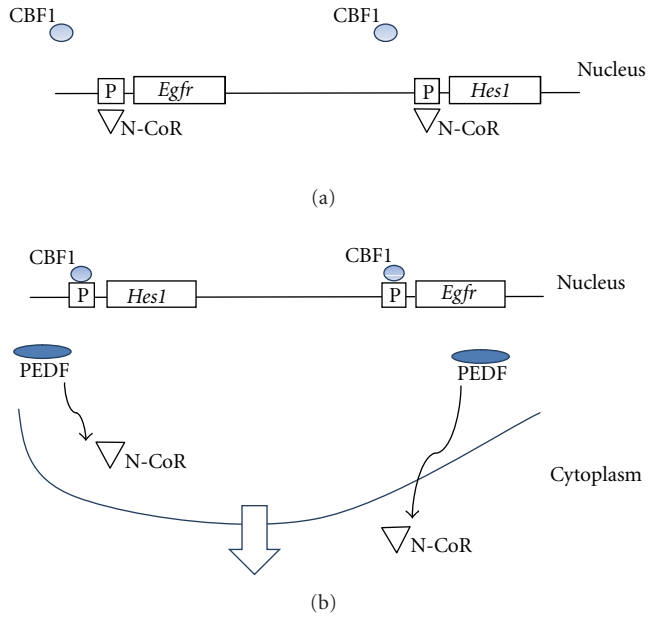

Figure 2

inhibitor of angiogenesis is the cause. It has been suggested that PEDF may cause regression of CNV promoted by VEGF [38]. The effects of PEDF on CNV are complicated: while PEDF suppresses the growth of endothelial cells exposed to fibroblast growth factor-2 (FGF2), it evokes proliferation of endothelial cells in the presence of high VEGF levels. In another report, PEDF showed concentration-dependent effects on CNV and endothelial cell function: low doses are inhibitory, but high doses can increase neovascularisation [39].

In a study by Hou et al. (2010), it was observed that injecting MSCs transduced with adenoviral vectors expressing PEDF (AdPEDF) caused a regression in neovascularisation as a result of the CNVs being encapsulated in retinal pigment epithelial cells (RPEs) [37].

Deterioration of photoreceptor cells may be caused by a continuous degeneration of retinal pigment epithelium (RPE) cells and can lead to vision loss. Adenovirally transduced bone marrow stromal (stem) cells carrying the PEDF gene exhibited the typical morphologies of RPE cells *in vitro*. Furthermore, when these cells were injected into the superior subretinal space of Royal College of Surgeons (RCS) rats, a well-established model of retinal degeneration due to defective phagocytosis of photoreceptor outer segments by the RPE, these cells produced more pronounced rescue effects than nontransduced cells. This suggests that PEDF has potency to protect photoreceptor cells from degeneration [40].

Stem cell therapy, especially using MSCs, has been considered as a promising method for treating myocardial infarction (MI) [41]. In a study where myocardial infarction was induced in C57BL/6 mice, it had been observed that MSCs could ameliorate MI injury, but that MSCs derived from older donors had less efficacy. It was found that older

MSCs secreted higher levels of PEDF than younger ones. Furthermore, infarcts treated with (AdPEDF-) transduced MSCs that over-expressed PEDF contained fewer ECs, VSMCs, and macrophages but had increased number of fibroblasts [42]. They concluded that it was the increased expression of PEDF in aged MSCs that impaired their therapeutic efficacy.

3. Conclusion

PEDF has a range of functions in different tissues and cells; however, the impact of this interesting protein on stem cells is not yet clear. PEDF expressed in feeder cells appears to have a supportive effect on stem cells such as hESCs and RPCs. PEDF affects NSCs via the Notch transcriptional pathway, promoting self-renewing divisions, maintaining a multipotent state of these cells and supporting expansion of the stem cell niche. Stem cells transduced with adenoviral vectors carrying PEDF exhibit potential for enhanced stem cell therapy in diseases such as macular degeneration. However, the effects of PEDF on stem cells in other disease states are clear. Whilst PEDF generally causes regression of CNV established by VEGF, the effects may be concentration dependent—both on the concentration of VEGF and PEDF. As for myocardial infarction, the presence of excessive PEDF is actually reported to reduce the efficacy of stem cell therapy. To conclude, the role of PEDF in stem cells in various pathologies does differ, and while counterproductive in some cases such as myocardial infarction, it may have a positive role to play in some instances. Further studies on PEDF and stem cells will help illuminate such instances and whether they can be used therapeutically.

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Review Article

PEDF in Diabetic Retinopathy: A Protective Effect of Oxidative Stress

Xiao-feng Zhu and Hai-dong Zou

Department of Ophthalmology, Shanghai First People's Hospital, Shanghai Jiao Tong University, Shanghai 200080, China

Correspondence should be addressed to Hai-dong Zou, zouhaidong@hotmail.com

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Diabetic retinopathy (DR) is a major cause of blindness in working age adults, and oxidative stress plays a vital role in the pathogenesis of DR. Pigment-epithelium-derived factor (PEDF), a multifunctional protein, has shown to inhibit the development of DR by accumulating evidence. This paper highlights the current understanding of probable mechanism about how PEDF blocks the deterioration of DR through its antioxidative properties and application prospects of PEDF as a novel therapeutic target in DR. Gene therapy of PEDF is becoming more and more acceptable and will widely be applied to the actual treatment in the near future.

1. Introduction

Diabetic retinopathy (DR) is the leading cause of acquired vision loss among adults of working age in developed countries worldwide and has been perceived as the most common microvascular complications of diabetes [1–3]. Therefore, within three to five years after the onset of type 1 diabetes and shortly after the diagnosis of type 2 diabetes, the diabetic patients are recommended to be screened for retinopathy with an initial dilated and comprehensive eye examination by an ophthalmologist regularly [4]. Chronic hyperglycemia which is well documented is a primary initiator of DR [5]. In principle, intensive glycemic control can delay the development of DR [5]. However, it is noteworthy that some patients may still develop DR even with good glycemic control [6, 7]. This remaining effect, prior to glycemic control, suggests a "metabolic memory" phenomenon. Furthermore, a growing number of studies have shown that the retina experience increased oxidative damage continuously, even in tight glycemic control, and oxidative stress plays a vital role in the pathogenesis of DR [6, 8].

The molecular mechanisms of hyperglycemia-induced DR are not fully clear, and the majority of publications focus on multiple biochemical pathways, including the augmentation of polyol pathway [9], protein kinase C (PKC) activation [10], increased advanced glycation endproducts (AGEs) formation, the receptor for AGEs and its activating ligands [11], and overactivity of the hexosamine pathway [12]. However, all the mechanisms are activated by a single event: aberrant production of the mitochondria-derived reactive oxygen species (ROS) to increase the level of oxidative stress [13]. Therefore, antioxidant therapy is being studied to prevent induction of the various pathogenic mechanisms of DR. With the failure of demonstrating that dietary supplementations of multiantioxidants have beneficial effects clinically [14], an antioxidant which could specifically target pathogenesis of DR is no time to delay. Pigment-epithelium-derived factor (PEDF), a 50-kDa secreted glycoprotein, recently is shown to inhibit the development of DR through its antioxidative properties. This paper summarizes the probable protective mechanism of PEDF in high-glucose-induced oxidative stress and application prospects of PEDF as a novel therapeutic target in DR.

2. PEDF and Its Potential Protective Role of Oxidative Stress in Diabetic Retinopathy

2.1. PEDF and Its Biological Function. PEDF is a 418-aminoacid, 50-KDa protein which was first purified from

conditioned medium from both fetal and adult retinal pigment epithelial (RPE) cells [15–18] and it is a noninhibitory member of the serine protease inhibitor (serpin) family. PEDF is widely expressing throughout the body, especially in the nervous system and the retina. According to current research, PEDF has shown that it is a multifunctional protein with demonstrable neurotrophic [19, 20], antiangiogenic [16], antivasopermeability [21], antiinflammatory [22], antifibrosis [23], and antitumorigenic [24] properties and inhibited the development of DR through its antioxidative properties by accumulating evidence [25–28].

Earlier clinical studies demonstrated an inverse correlation of the levels of intraocular PEDF and the development of abnormal angiogenesis in some ocular diseases, such as proliferative diabetic retinopathy (PDR) [16, 29–32]. Similarly, in mouse model of type 2 diabetes mellitus, lower vitreous or aqueous humour levels of PEDF are associated with early phase of experimental DR [16, 31, 33], and decreased protein levels of PEDF in the retina are associated with ischemia-induced retinal neovascularization in the oxygen-induced retinopathy (OIR) model as well [34].

Therefore, existing research data demonstrated that upregulation or substitution of PEDF may be a promising therapeutic target for DR [16, 25, 35–37], especially oxidative stress-involved retinal tissue damage [38]. Then, experimental interventions to increase locally PEDF concentrations, either by an adenovirus expressing human PEDF or a purified recombinant PEDF protein, have shown to attenuate retinal tissue damage in different animal models [22, 39]. Thus, finding a good way for controlling PEDF expression and action in the retina has become a research focus.

In addition, PEDF suppresses vascular endothelial growth-factor-induced (VEGF-induced) retinal microvascular endothelial cell proliferation and migration [40] and inhibits VEGF activation in human retinal endothelial cells in vitro condition [41]. In contrast, PEDF has a synergistic action with VEGF on cell proliferation in endothelial cells cultured in the presence of VEGF [42]. Moreover, the VEGF and PEDF expression in Müller cells is unbalanced under high-glucose concentration, which contributes to retinal neovascularization in DR [43]. Since VEGF plays a pivotal role in the formation of ROS, to defining the specific effects of PEDF, high-glucose-induced oxidative stress will also be important.

2.2. Antioxidative Properties of PEDF in Diabetic Retinopathy

2.2.1. Inhibit AGEs-Induced Injury. AGEs, senescent macroproteins formed at an accelerated rate in diabetes, cause apoptotic cell death in retinal pericytes [44, 45]. Studies have found AGEs significantly decreased endothelial mRNA levels of PEDF in endothelial cells [46], and PEDF proteins protect cultured retinal pericytes from AGEs-induced injury probably via oxidative stress generation [25]. Thus, we will introduce the different possible mechanisms of PEDF to inhibit AGEs-induced injury in DR.

Retinal Pericytes. Earlier studies have found that loss of pericytes and increased vascular permeability, followed by microvascular occlusion in the retinas, ultimately leaded to the development of DR [47, 48]. Experimental analysis revealed that pericytes possessed of a membrane protein with binding affinity for PEDF which significantly inhibited AGEs-induced ROS generation and the subsequent decrease in DNA synthesis and apoptotic cell death in pericytes. Furthermore, PEDF proteins completely restored the downregulation of bcl-2 (an antiapoptotic molecule) gene expressing in AGEs-exposed pericytes [25]. Similarly, the studies have demonstrated that PEDF completely blocked high-glucose- or H_2O_2-induced intracellular ROS generation and an increased ratio of bax to bcl-2 mRNA level with subsequent activation of caspase-3 in pericytes [49]. In addition, PEDF protected high glucose- (30 mM) or H_2O_2-induced pericyte apoptosis and dysfunction through its antioxidative properties via glutathione peroxidase (GPx) induction. Simultaneously, the study also found that PEDF's mRNA levels themselves were downregulated in high-glucose (HG-) or H_2O_2-exposed pericytes.

These results all demonstrated that PEDF proteins protected cultured pericytes from AGEs-induced cytotoxicity through its antioxidative properties, and substitution of PEDF proteins may be a promising strategy in treatment of patients with DR.

Monocyte Chemoattractant Protein-1 (MCP-1). Further work have shown that PEDF prevented the AGEs-induced upregulation of monocyte chemoattractant protein-1 (MCP-1) mRNA contents as well as protein production in microvascular endothelial cells (ECs) [50]. Moreover, levels of MCP-1 in vitreous fluids have been correlated with the severity of PDR [51].

Rage. There is a growing body of evidence that RAGE is a signal-transducing receptor for AGEs, and that engagement of RAGE by AGEs elicits vascular inflammation and alters gene expression in retinal vascular wall cells, thereby it is involved in the development and progression of DR [45, 50, 52–54]. Recent studies have shown that PEDF could inhibit diabetes- (in the eye of diabetic rats) or AGEs-induced (in vitro) RAGE gene expression by blocking the superoxide-mediated NF-kappaB activation [36].

Endothelial NO Synthase (eNOS). Studies have shown that PEDF prevented the AGEs-elicited endothelial NO synthase (eNOS) reduction through its antioxidative properties in AGEs-exposed human umbilical vein ECs (HUVECs) [55]. And endothelial dysfunction due to reduced synthesis and/or bioavailability of nitric oxide (NO) is an initial step of atherosclerotic vascular disease in diabetes [56–58].

Platelet Activation and Aggregation. There is accumulating evidence that the oxidative stress generation is involved in platelet activation and aggregation [59, 60]. These observations suggest that the inhibition of platelet activation and aggregation may be a novel therapeutic target for preventing the development and progression of vascular complications in patients with diabetes. Further, the researchers have found

that PEDF prevented platelet activation and aggregation in diabetic rats or AGEs-injected rats through its antioxidative properties by suppressing NADPH oxidase-driven superoxide generation, deleterious effects of AGEs [61].

The Src Pathway. PEDF inhibited AGEs-induced ROS generation by increasing levels of SOD and GSH and also blocked the activation of caspase-3. Furthermore, PEDF induced cell survival via the Src pathway by Src phosphorylation at Y419, as evidenced by a pharmacological inhibitor and Src mutants [62].

Phosphatidylinositol 3-Kinase (PI3K)/Akt Pathway. Recent studies demonstrated that PEDF could inhibit the AGEs-BSA-induced permeability via phosphatidylinositol 3-kinase (PI3K)/Akt pathway. AGEs-BSA increased the ECs permeability by stimulating ROS generation via NADPH oxidase activity and Akt phosphorylation at Ser473. PEDF decreased ROS generation in AGEs-BSA-exposed endothelial cells by suppressing the NADPH oxidase activity via downregulating the phosphorylation of $p22^{PHOX}$ at Thr147. This led to blockade of AGEs induction of PI3K/Akt activation in permeability. Furthermore, PEDF inhibited the AGEs-BSA-induced permeability by increased expression of tight junction protein zona occludens-1 (ZO-1), coincident with an increase in barrier properties of endothelial monolayer [63].

2.2.2. Inhibit Leptin-Induced Injury. PEDF was found to inhibit the leptin-induced ROS generation and upregulation of VEGF mRNA levels including any increase in DNA synthesis in microvascular ECs [64]. Indeed, leptin levels in vitreous were correlated with PDR [65].

2.2.3. Inhibit Tumor Necrosis Factor-α (TNF-α-) Induced Injury. The studies demonstrated that PEDF inhibited tumor necrosis factor-α (TNF-α-) induced redox-sensitive transcriptional factor NF-kappaB activation and subsequent interleukin IL-6 overexpression at both mRNA and protein levels in human umbilical vein endothelial cell (HUVEC) by suppressing NADPH oxidase-mediated ROS generation [66]. In addition, TNF-alpha which is initially involved in the pathogenesis of atherosclerosis [67, 68] and the classic proinflammatory cytokines, downregulated PEDF mRNA levels [66].

2.2.4. Inhibit Angiopoietin-II (Ang II-) Induced Injury. The researchers have found that Angiopoietin-II (Ang II) significantly induced NF-kappaB activation and subsequent MCP-1 expression in HUVEC, both of which were completely inhibited by PEDF. Subsequently, PEDF inhibited Ang-II-induced upregulation of mRNA levels of $p22^{PHOX}$, Nox4, and $gp91^{PHOX}$/Nox2, which are membrane components of NADPH oxidase and its enzymatic activity in HUVEC [69]. Another study found that Ang II also significantly decreased PEDF mRNA levels in ECs, which was completely reversed by an Ang II type 1 receptor blocker, telmisartan [70]. Furthermore, anti-PEDF Ab significantly inhibited the growth-stimulating effects of cocultured ECs on pericytes. These results demonstrated that PEDF, an EC-derived mitogen or survival factor for retinal pericytes, inhibited Ang-II-induced ECs activation by suppressing NADPH-oxidase-mediated ROS generation, and suppression by Ang II of the EC-derived PEDF may be involved in exacerbation of DR in patients with hypertension. In addition, PEDF was found to completely inhibit high-glucose- or H_2O_2-induced increase in a mRNA ratio of Ang II to Angiopoietin-I (Ang I) and upregulation of VEGF mRNA levels in pericytes. VEGF and angiopoietin (Ang) have been known that they were the major regulators of vascular integrity and involved in DR as well [71].

2.2.5. Inhibit HOL-LDL-Induced Injury. PEDF ameliorated HOL-LDL-induced MCP-1 and the subsequent NF-kappaB activation effectively. Moreover, PEDF significantly ameliorated HOG-LDL-induced ROS generation through upregulation of superoxide dismutase 1 expression [72]. This study represented a new mechanism for the salutary effect of PEDF in DR.

2.2.6. Inhibit High-Glucose (HG)-Induced JAK2/STAT3 Activation. A recent report suggested that ACEI exerted a protective effect on DR, and this protective effect could be reflected by a decreased VEGF-to-PEDF ratio, which is a result of reduced mitochondrial ROS production itselfs caused by ACEI-induced increase of proliferator-activated receptor gamma (PPARγ) and subsequent upregulation of uncoupling protein-2 (UCP-2) expression [73]. Further work in vitro has demonstrated that PEDF could decrease mitochondria-derived ROS generation and subsequently downregulate VEGF expression, possibly through inhibiting HG-induced JAK2/STAT3 activation [74]. These studies pave a new way for future in treatment of DR.

2.2.7. Regulation of PEDF Expression In Vivo. PEDF levels in aqueous humor or vitreous were associated with total antioxidant capacity in humans [38, 75] and suggested that PEDF may act as an endogenous antioxidant in the eye and upregulation or substitution of PEDF may be a therapeutic target for oxidative stress-involved eye diseases, especially PDR.

Retinal PEDF levels were reduced in diabetic rat, which were restored by PEDF injections. Decreased amplitudes of a- and b-wave in the ERG in diabetic rats, which were in parallel with GFAP overexpression in the Müller cells, also could be blocked by PEDF injections. Further, retinal 8-OHdG, $p22^{PHOX}$, VEGF levels, and NADPH oxidase activity were increased, and BRB was broken in diabetic rats, both of which were ameliorated by the treatment of PEDF [26].

In addition, studies with rats, intravenous administration of AGEs, and simultaneous treatments with PEDF demonstrated that PEDF decreased ROS generation in AGEs-exposed endothelial cells by suppressing NADPH oxidase activity via downregulation of mRNA levels of $p22^{PHOX}$ and $gp91^{PHOX}$ and inhibited the AGEs-induced vascular

hyperpermeability, the characteristic feature of early DR, by suppressing VEGF expression [76].

Furthermore, the action of PEDF not only varied with the cell type but also depended on its concentration and environmental conditions [77].

3. Application Prospects of PEDF in Diabetic Retinopathy

Large prospective clinical studies have shown that intensive blood glucose control reduced the incidence and progression of DR [5, 48]. However, strict control of hyperglycemia is often difficult to maintain and may increase the risk of severe hypoglycemia in diabetic patients. In addition, photocoagulation and vitrectomy, current conventional therapeutic options for the treatment of PDR, are limited by considerable side effects. Therefore, developing novel therapeutic strategies that specifically target pathogenesis of DR is desired for patients with diabetes. Based on the above role and regulation mechanism of PEDF in DR, the research on the treatment of PDR has turned to the regulation of angiogenesis inhibitors and growth factors.

3.1. Current and Potential Molecular Therapie of PEDF

3.1.1. PEDF and PEDF-Derived Peptide. The multifunctional PEDF, more effective than other antiangiogenic factors, is a good candidate for treatment of DR. Because a large part of endogenous PEDF-binding affinity to extracellular matrix components and cell-surface receptors, it would not achieve a protective effect. However, injection of exogenous PEDF, competitive binding of PEDF in the extracellular binding sites, will release PEDF from extracellular matrix and subsequently achieve therapeutic concentrations. The problems of current stage are as follow: (1) PEDF is a 50-kDa protein, and its structural features limit practical application as pharmaceuticals; (2) underestimated the carrier itself could induce the inflammatory response; (3) currently there is no good control methods for PEDF which has secreted into the extracellular. However, many biologically active fragment of PEDF has been known, and if we can find the active fragment, equivalent or near equivalent with PEDF, which is replaced with small peptides, we will avoid or reduce the inflammatory reaction by virus or other carriers through slow release of directly permeating sclera or intravitreal injection [78]. A recent study demonstrated that an antiangiogenic peptide, PEDF-34, reduced circulating endothelial cells during ischemia-induced neovascularization [79]. Thus, the PEDF-34 peptide could be a superior biological therapeutic for the treatment of PDR and has great potential for large-scale pharmaceutical development. A disadvantage, however, of using small peptide derivatives is that they tend to be cleared rapidly from tissues and thus may be less effective therapeutically unless they are protected from rapid enzymatic degradation and tissue clearance.

3.1.2. Biodegradable Nanospheres. Nanospheres, biocompatible, biodegradable, and producer of fewer side effects, is one way to avoid disadvantage of peptide. Moreover, there is abundant experimental evidence that intravitreal injections of drugs encapsulated in PLGA-poly (lactide-co-glycolide) nanospheres are both useful and effective [80, 81]. A recent study has found that delivering $PEDF_{82-121}$, a free peptide, in PLGA nanospheres is an effective way of achieving controlled release of therapeutically active levels of the peptide [82]. Such delivery systems can be manipulated to provide controlled release of physiological levels of bioactive products for both short- and long-term needs [83].

3.1.3. Gene Therapy. Gene therapy involves the replacement of a faulty gene or the insertion of a new gene. Treatment of angiogenic disease can be achieved by the insertion of genes that encode antiangiogenic proteins, including the vitreous body and subretinal injection. Adenovirus and adeno-associated virus (AAV) has shown particular promise in the delivery of antiangiogenic DNA. The first human trial of recombinant PEDF introduced via the adenoviral vector AdPEDF.11 suggested that intravitreal delivery is relatively well tolerated with the antiangiogenic effect of PEDF persisting for several months [84]. PEDF transgenic (PEDF-Tg) mice that ubiquitously express human PEDF driven by the β-actin promoter inhibited neovascular disorders such as DR [85]. These results have provided valuable information with regards to gene therapy for the treatment of DR.

4. Conclusion

PEDF, a multifunctional factor, has shown to inhibit the development of DR by accumulating evidence. This paper highlights the current understanding of probable mechanism about how PEDF blocks the deterioration of DR through its antioxidative properties and application prospects of PEDF as a novel therapeutic target in DR. Gene therapy is becoming more and more acceptable and widely applied to the actual treatment. PEDF and its fragments, transferred into virus vector or made of biodegradable implant for local injection, have broad application prospects in treatment of DR. However, the drug screening and selection of the best way to enter the human body need large number of researches.

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Research Article

PEDF and VEGF-A Output from Human Retinal Pigment Epithelial Cells Grown on Novel Microcarriers

Torsten Falk,[1] Nicole R. Congrove,[2] Shiling Zhang,[1] Alexander D. McCourt,[1] Scott J. Sherman,[1,3] and Brian S. McKay[2,4]

[1] *Department of Neurology, College of Medicine, The University of Arizona, AHSC, P.O. Box 245023, 1501 N. Campbell Avenue, Tucson, AZ 85724, USA*
[2] *Department of Ophthalmology & Vision Science, The University of Arizona, Tucson, AZ 85724, USA*
[3] *Department of Physiology, The University of Arizona, Tucson, AZ 85724, USA*
[4] *Department of Cellular and Molecular Medicine, The University of Arizona, Tucson, AZ 85724, USA*

Correspondence should be addressed to Torsten Falk, tfalk@u.arizona.edu

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Human retinal pigment epithelial (hRPE) cells have been tested as a cell-based therapy for Parkinson's disease but will require additional study before further clinical trials can be planned. We now show that the long-term survival and neurotrophic potential of hRPE cells can be enhanced by the use of FDA-approved plastic-based microcarriers compared to a gelatin-based microcarrier as used in failed clinical trials. The hRPE cells grown on these plastic-based microcarriers display several important characteristics of hRPE found *in vivo*: (1) characteristic morphological features, (2) accumulation of melanin pigment, and (3) high levels of production of the neurotrophic factors pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor-A (VEGF-A). Growth of hRPE cells on plastic-based microcarriers led to sustained levels (>1 ng/ml) of PEDF and VEGF-A in conditioned media for two months. We also show that the expression of VEGF-A and PEDF is reciprocally regulated by activation of the GPR143 pathway. GPR143 is activated by L-DOPA (1 μM) which decreased VEGF-A secretion as opposed to the previously reported increase in PEDF secretion. The hRPE microcarriers are therefore novel candidate delivery systems for achieving long-term delivery of the neuroprotective factors PEDF and VEGF-A, which could have a value in neurodegenerative conditions such as Parkinson's disease.

1. Introduction

Parkinson's disease (PD) is the 2nd most common neurodegenerative disease. Risk for PD is primarily related to increased age, and the most vulnerable neurons are the dopaminergic neurons in the substantia nigra. Destruction of these neurons leads to a disruption of the pathway between the substantia nigra and striatum and produces a severe dopamine deficiency [1]. Dopamine plays an important role in the communication between the thalamus, striatum, and cortex. Reduction of dopamine levels leads to the cardinal motoric features of Parkinson's disease: tremor, generalized slowness of movement, and difficulty transitioning from one movement to the next [1]. The most effective form of treatment for PD is dopamine replacement therapy.

The two most pressing therapeutic challenges in PD are to (1) provide a stable level of dopamine replacement and (2) slow or halt disease progression as reviewed [2–4]. Pharmacological treatment of PD is satisfactory in the early stages of the disease but becomes problematic as the disease progresses. Treatment with the potent dopamine precursor L-DOPA is eventually required for almost all patients, and its erratic absorption and short half-life lead to the development of disabling fluctuations in the treatment response [5]. Providing a continuous intracerebral source of dopamine by using cell-based therapy has been at the front line of experimental efforts [6]. One strategy has been to use hRPE cells as a local source of L-DOPA [7–11]. Cell transplantation of hRPE using cross-linked gelatin microcarriers has been used in preclinical and clinical studies of PD and appears to be a

safe technique [8, 12, 13]. However, the therapeutic potential for this platform has been questioned due to the recently reported failure of the commercially sponsored Phase II clinical trial [13]. Among the several potential contributing factors to the lack of efficacy, we would like to point out three specifically. Firstly, there may have been a failure to achieve long-term survival of the hRPE monolayers on the gelatin-based microcarriers. Immune system responses to the graft may have been responsible for graft failure, and these could be ameliorated by the use of immunosuppression in future trials or avoided by the use of autologously produced hRPE cultured from a small biopsy. Secondly, long-term survival of the graft may also be hindered by digestion of the gelatin-based microcarrier, needed to keep hRPE as a differentiated functional monolayer. Inert plastic materials might provide for a more stable microcarrier since these will not be degraded. Finally, the failure to account for the role of neurotrophic factors in the positive results seen in preclinical and Phase 1 trials could be an important aspect. The clinical benefit found in the initial trial and previously described in both rat and nonhuman primate models of PD [8, 14] was presumed to be due to the production of L-DOPA. However, some of the observed benefit could be related to the release of neuroprotective factors [15] such as pigment epithelium-derived factor (PEDF) [16, 17] and vascular endothelial growth factor-A (VEGF-A) [17]. Indeed, primate studies of hRPE transplantation have indicated a persistent clinical benefit despite little or no long-term survival of the initial engraftment as assessed by both behavioral and neuroimaging measures [18]. These data suggest that a neurorestorative effect outlasting survival of the cell transplant was induced. The Phase II trial design lacked screening of the cells for normal levels of production of PEDF or VEGF-A prior to implantation. We believe it would be a mistake to allow the result of the single Phase II trial to prematurely halt further development of the hRPE platform. To reinvigorate this field, other properties and new manipulations of hRPE cells need to be put forth. It is in this regard that we emphasize and further extend the important findings that (1) PEDF and VEGF-A are neurotrophic and neuroprotective factors in culture models of PD [17, 19], (2) the PEDF level of hRPE cells is subject to variability depending on the source and also the pigmentation level [20], and (3) the PEDF level is regulated by L-DOPA via the G-protein-coupled receptor GPR143 (OA1) [21]. Further investigations of the neurotrophic potential of hRPE cells, due to secretion of PEDF and VEGF-A, have the potential to revive interest in the hRPE cell platform as cell transplantation therapy for PD. However, further understanding of the regulation of these factors in hRPE cells will be required, particularly since L-DOPA is central to the strategy of dopamine replacement using hRPE transplantation. A reciprocal regulation of PEDF and VEGF-A is known in many body tissues [16, 17, 22]; for example, when VEGF-A levels are high, PEDF levels are generally low and vice versa. Since activation of GPR143 up-regulates PEDF production and secretion, it would be logical to investigate if there is also a reciprocal effect on VEGF-A.

Further investigation of the neurotrophic potential of hRPE cells should be coupled with research to aid in the development of microcarriers with increased long-term stability. Such studies would allow the development of more stringent criteria that could be used to screen hRPE cell lines and predict their likelihood of robust clinical efficacy. The experiments described here take a first step in this direction by (1) examining the levels of PEDF and VEGF-A secretion of hRPE on different types of plastic-based microcarriers and (2) investigating if there is an effect of L-DOPA on regulation of VEGF-A secretion as has been shown for PEDF secretion.

2. Materials and Methods

2.1. RPE Culture. RPE cells were isolated from human donor eyes obtained from Advanced Bioscience Resources as described [23, 24] and maintained in Chee's essential medium replacement media (CEM-RM). To seed the microcarriers, hRPE cells (1×10^6) were plated in a 12.5 cm^2 flask coated with 3% agar and gently rocked for 2 hours with 100 μL microcarriers delivered as a 1 : 1 ratio (v/v) microcarrier to media slurry, 200 μL total volume. The total number of microcarriers differed among the microcarrier types as the microcarrier size differed, but the packed volume of microcarriers was the same in each group. We used four types: the gelatin-based CultiSpher microcarriers (macroporous gelatin microcarriers, diameter: 130–380 μm; Percell Biolytica, Astorp, Sweden), as well as 3 plastic-based microcarriers from Solohill Engineering (Ann Arbor MI) that are free from animal products and FDA approved: Hillex II (modified polystyrene with cationic trimethyl ammonium, diameter: 160–180 μm), Plastic Plus (cross-linked polystyrene, cationic charged, diameter: 90–150 μm), and ProNectin F (trademark Solohill, polystyrene coated with recombinant RGD containing protein, diameter 90–150 μm) microcarriers.

2.2. PTU + L-DOPA Treatment. To determine whether GPR143 signaling activity controlled VEGF-A secretion, we used a strategy similar to that which illustrated the receptors control over PEDF [21]. hRPE cells were plated into 24-well plates in CEM and maintained at confluency for 3-4 months to facilitate differentiation of the monolayers [23]. Because L-DOPA is the endogenous ligand for GPR143, and all pigmented cells produce L-DOPA, we used 200 μM phenyl thiourea (PTU) to inhibit the endogenous production of L-DOPA by tyrosinase, which then downregulates the GPR143 signaling pathway, allowing us to control the signaling pathway and use a defined L-DOPA concentration. In the presence of PTU, we then added 1.0 μM L-DOPA to stimulate the GPR143 signaling pathway. Each treatment was on the cells for 72 hours, after which the media was collected and immediately frozen at -80°C.

2.3. Photomicrography. Images were acquired digitally on an Olympus IX70 inverted microscope and camera using Olympus MagnaFire software using a 20x objective (400x magnification).

2.4. ELISA Measurement of VEGF-A, PEDF, GDNF, and BDNF. Enzyme-linked immunosorbent assays (ELISAs) were used to determine the concentrations of VEGF-A (R&D Systems, Minneapolis, MN, USA), PEDF (Chemicon International, Temecula, CA, USA), glial-cell-line-derived neurotrophic factor (GDNF; Promega, Madison, WI, USA) and brain-derived neurotrophic factor (BDNF; Promega, Madison, WI, USA) in conditioned medium from hRPE cells grown on microcarriers or normal tissue culture flasks. The medium was harvested from the hRPE cultures every 3-4 days to produce the conditioned medium (CM) samples over long-term time courses. The CM was frozen at −80°C immediately after harvest. The ELISAs were performed in duplicate as directed by the manufacturers. ELISA results were obtained using an EMAX Microtiter Plate Reader (Molecular Devices, Downingtown PA, USA).

3. Results

3.1. Growth of hRPE on Microcarriers. The hRPE cells were seeded onto the microcarriers and followed for 2 months in culture. Figure 1 shows example photomicrographs of the different plastic-based microcarriers covered with monolayers of hRPE at 1 and 2 months after seeding. Figures 1A and 1B depict hRPE cells on Hillex II microcarriers, at 1 month and 2 months. The hRPE formed a clearly visible monolayer around the microcarrier with the characteristic melanin pigment and the polygonal morphology reminiscent of the normal histological features found *in vivo* [23]. Figures 1C and 1D depict hRPE cells on Plastic Plus microcarriers, and Figures 1E and 1F depict hRPE cells on ProNectin F microcarriers. From the visual inspection, the Hillex II microcarriers appear to have the most uniform monolayer, but all microcarriers had significant clumping. The hRPE cells remained as a monolayer on the Hillex II microcarrier most frequently. The hRPE cells on the Plastic Plus microcarriers generally appeared as an amorphous mass of cells and microcarriers as illustrated. The hRPE cells on the ProNectin F microcarriers were generally present as a monolayer but not as regular as on the Hillex II microcarriers.

3.2. PEDF Concentration Time Course. The PEDF present in hRPE media was measured in a series of ELISAs. In all, the ELISA results show a significant PEDF secretion into the medium by hRPE cells over 2 months.

For each of the four types of microcarrier with monolayers of hRPE cells, the PEDF concentration was measured for samples collected every few days for 2 months, as shown in Figure 2. The cells on the CultiSpher microcarrier secreted insignificant amounts of PEDF. This was explained by the inability of the CultiSpher to create a sufficient base for hRPE cells to form a healthy monolayer. The other three microcarrier types, Hillex II, Plastic Plus, and ProNectin F all cultured with hRPE from the same donor eye, provided comparable PEDF levels. After a short rising phase, maximal expression levels were reached at about 3-4 ng/mL from the Hillex II microcarriers and about 2 ng/mL from Plastic Plus and ProNectin F microcarriers, and the PEDF concentration remained stable for the duration of the experiment (2 months) as shown in Figure 2.

3.3. VEGF-A Concentration Time Course. The VEGF-A present in hRPE media was measured in a series of ELISAs. In all, the ELISA results show a significant VEGF-A secretion into the medium by hRPE cells over time.

For each of the four types of microcarrier with monolayers of hRPE cells, the VEGF-A concentration was measured for samples collected every few days for 2 months, as shown in Figure 3. The cells on the CultiSpher microcarrier secreted insignificant amounts of VEGF-A, which again was attributed to largely unsuccessful growth of cultures of hRPE cells on CultiSpher over time. The other three microcarrier types Hillex II, Plastic Plus, and ProNectin F, provided comparable VEGF-A levels. After a rising phase of about 20 days, stable expression levels were reached at about 15 ng/mL, and the VEGF-A concentration remained stable for the duration of the experiment (2 months) as shown in Figure 3. The higher variability within the PEDF versus the VEGF-A time course is explained by the fact that the PEDF concentration measured is much closer to the detection threshold than the VEGF-A concentration.

3.4. GDNF and BDNF Concentration Time Course. The GDNF present in hRPE media was measured in a series of ELISAs. In all, the ELISA results showed a significant GDNF secretion into the medium by hRPE cells grown on Hillex II, Plastic Plus, and ProNectin F microcarriers only over the first 3 weeks as depicted in Figure 4. After day 22 the GDNF level had dropped below the detection threshold. Even at the start, the GDNF concentration was only in the pg/mL range, compared to VEGF-A and PEDF for which ng/mL concentrations were measured.

We also examined if BDNF was present in hRPE media as measured in a series of ELISAs. In all, the ELISA results showed that the hRPE cells did not secrete any measurable BDNF into the medium on all microcarriers tested at any time point (data not shown).

3.5. Regulation of Neurotrophic Output of hRPE. We have previously shown that GPR143 signaling regulates PEDF production in hRPE cells [21]. To extend this finding, we investigated regulation of VEGF-A output in hRPE cells, using the tissue-type, stable, pigmented monolayer for a minimum of 3 months prior to experiment initiation. We used the same paradigm used by Lopez et al. 2009 [21] to define PEDF regulation in hRPE cells. The tyrosinase inhibitor PTU was used to block endogenous L-DOPA production and allow control of OA1 signaling without complicated endogenous activity. We added 200 μM PTU + 1 μM L-DOPA which caused a significant decrease in VEGF-A production that was restored to baseline when L-DOPA was removed. This decrease of VEGF-A production after addition of L-DOPA compared to the untreated cells ($P < 0.0005$, paired t-test with Bonferroni Correction, $n = 9$) is shown in Figure 5. After the L-DOPA was removed, there was

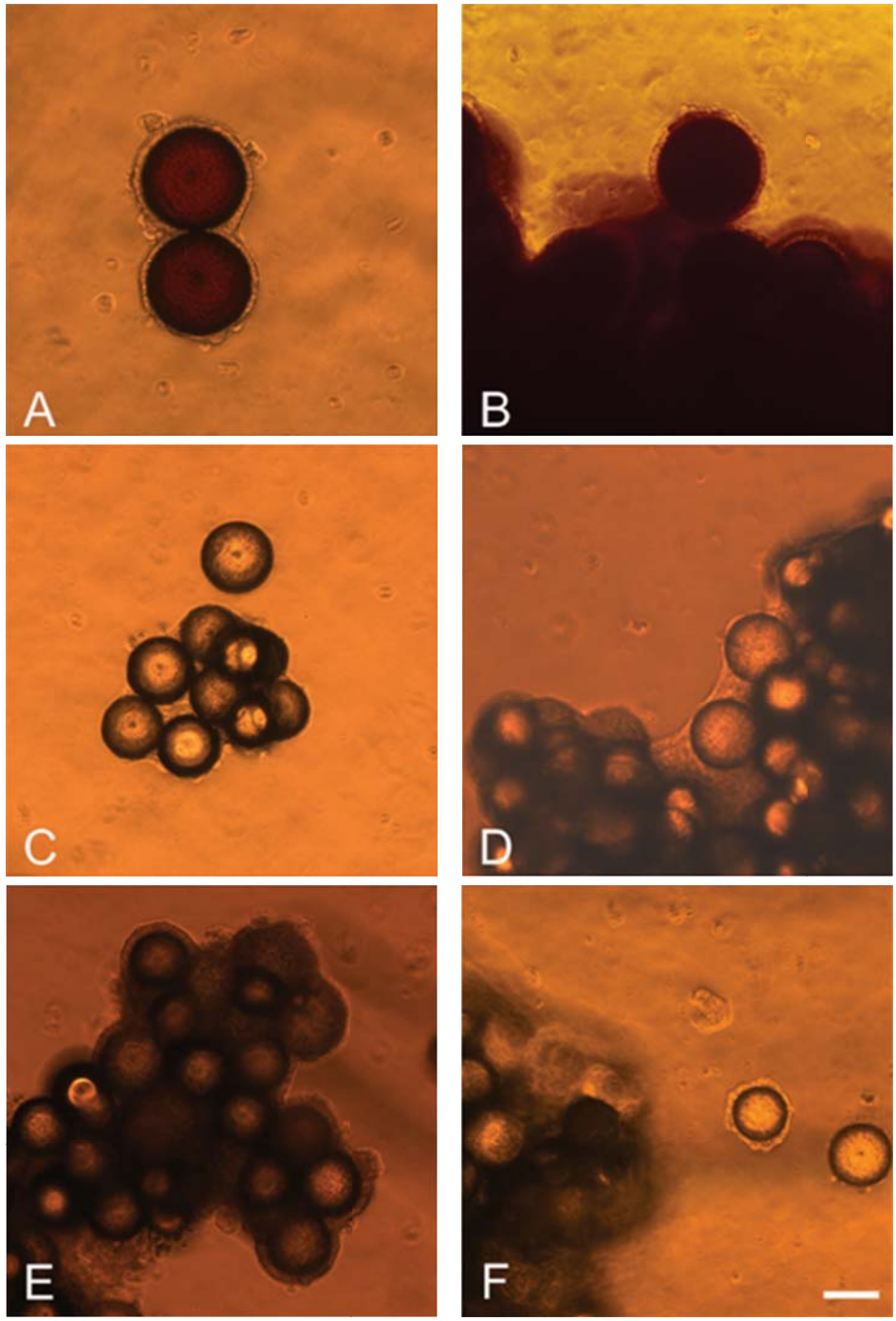

Figure 1: Example photomicrographs for hRPE cells grown on different plastic-based microcarrier types at 1 month (A, C, E) and two months (B, D, F). Hillex II microcarriers are shown in A and B, Plastic Plus microcarriers are shown in C and D, and ProNectin F microcarriers are illustrated in E and F. Note the characteristic melanin pigment and the polygonal morphology reminiscent of the normal histological features found *in vivo*. Scale bar = 100 μm.

a significant increase of VEGF-A production ($P < 0.0005$, paired t-test with Bonferroni Correction, $n = 9$) by the cells, returning toward baseline. The comparison of the cells at baseline and after the washout period (paired t-test with Bonferroni's correction, $n = 9$) shows no significant change in VEGF-A concentration.

4. Discussion

RPE cells have been studied as a transplantation platform for the treatment of PD based on their ability to produce L-DOPA as intermediate in the melanin synthesis pathway and thus provide a source of continuous intracerebral dopamine

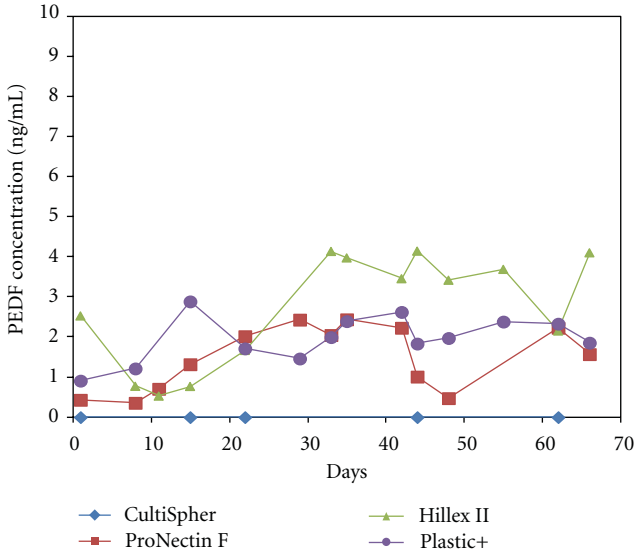

Figure 2: PEDF concentration time course. The PEDF concentration in the media from the hRPE cells on the different microcarriers was measured with an ELISA. After a short rising phase the PEDF concentration reached a plateau. The hRPE cells on Hillex II, Plastic Plus, and ProNectin F microcarriers secreted comparable amount of PEDF, whereas hRPE cells on the CultiSpher microcarriers did not secrete significant amounts of PEDF.

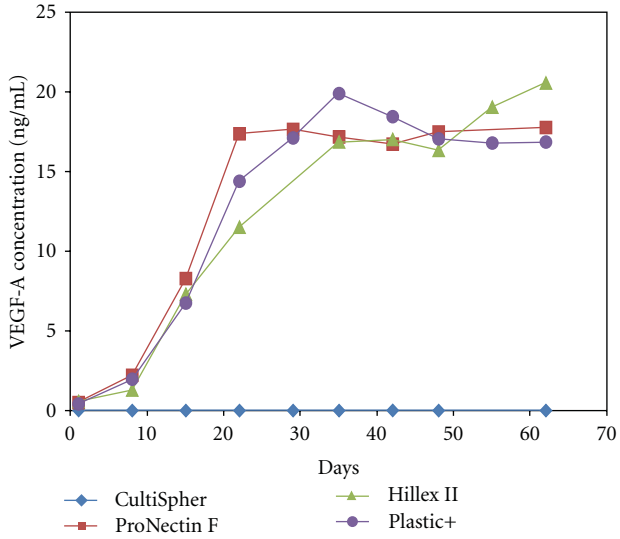

Figure 3: VEGF-A concentration time course. The VEGF-A concentration in the media from the hRPE cells on the different microcarriers was measured with an ELISA. After a rising phase in the first 20 days after seeding the VEGF-A, concentration reached a plateau and remained stable until the end of the experiment. The hRPE cells on Hillex II, Plastic Plus, and ProNectin F microcarriers secreted comparable amount of VEGF-A, whereas hRPE cells on the CultiSpher microcarriers did not secrete significant amounts of VEGF-A.

replacement. In their normal anatomical location, RPE cells function to facilitate retinal survival and activity, in part by the secretion of the neurotrophic factors PEDF and VEGF-A [17, 25, 26]. These factors could potentially provide an additional benefit in PD by virtue of neuroprotective effects on dopaminergic neurons. Both of these molecules have been extensively studied with regard to their role in controlling tissue vascularity, where they have opposing effects to either, respectively, inhibit or promote angiogenesis [16, 17, 26]. In addition to their role in controlling angiogenesis, recent studies have supported an important neurotrophic function for both molecules in the CNS where they may work in concert [17].

Neuroprotective action of PEDF has also been described in a variety of neuronal cell types grown *in vitro* as well as in a postnatal organotypic culture model of motor neuron degeneration [27, 28]. PEDF prevented the death and atrophy of spinal motor neurons *in vivo* in the developing neonatal mouse after axotomy [29]. Of importance for the current study, PEDF has been shown to be both neurotrophic and neuroprotective in two *in vitro* models of PD [19].

It has been shown that VEGF-A has neurotrophic and neuroprotective properties in explants of the ventral mesencephalon [30]. VEGF-A has been shown to cause axonal outgrowth on cultured ganglia in a dose-responsive manner [31]. At VEGF-A concentrations (10–50 ng/mL) comparable to the level found in hRPE-conditioned media in this study, neuroprotective effects have been observed on cultured cells subjected to hypoxia and glucose deprivation [32]. Unfortunately, at higher concentrations, because of its angiogenic nature, VEGF-A may cause increased permeability of the blood-brain barrier, which could be detrimental in causing cerebral edema or other untoward effects [33]. If hRPE cells are to be implanted in the brains of PD patients as a therapy, it will be important to establish that VEGF-A production is sufficient for neuroprotection but below the threshold for disruption of the blood-brain barrier.

The combined neurotrophic effects and the exact ratio of PEDF and VEGF-A could also be important considerations. A possible concerted neurotrophic effect by PEDF and VEGF-A in PD was suggested by Yasuda et al. [34] reporting a significantly positive correlation in the striatal levels of PEDF and VEGF-A in brains acquired at autopsy in PD patients. This study also monitored the striatal levels of PEDF relative to the VEGF-A levels in a rodent model following a toxic insult to the dopaminergic pathway. Acute damage to dopaminergic neurons induced a rise in PEDF levels in the CNS, supporting the hypotheses that PEDF acts as an endogenous natural neuroprotective response factor.

In this study we found that hRPE cells grown on plastic-based microcarriers retain their ability to produce both PEDF and VEGF-A, but we were unable to produce long-term cultures of hRPE cells on gelatin-based CultiSpher microcarriers. Thus, by virtue of the cells failing after initial attachment, we did not observe significant levels of either neurotrophic factor with those microcarriers. The hRPE cells transplanted in the Spheramine clinical trials utilized gelatin-based Spheramine microcarriers, and their potential to produce PEDF and VEGF-A was never investigated. The extent to which neuroprotection is operative in clinical trials

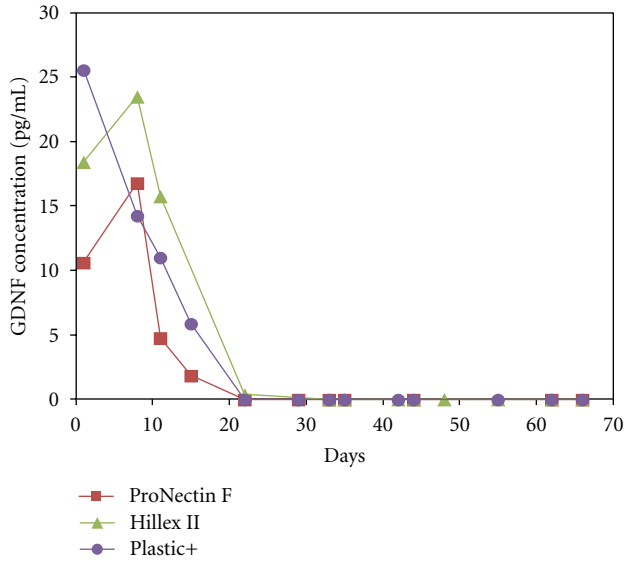

Figure 4: GDNF concentration time course. The GDNF concentration in the media from the hRPE cells on the different microcarriers was measured with an ELISA. The hRPE cells on Hillex II, Plastic Plus, and ProNectin F microcarriers secreted comparable amounts of GDNF at the start of the time course. The concentration was an order of magnitude less when compared to VEGF-A and PEDF. The GDNF concentration declined over the first 3 weeks, and there was no detectable amount of GDNF from day 22 on.

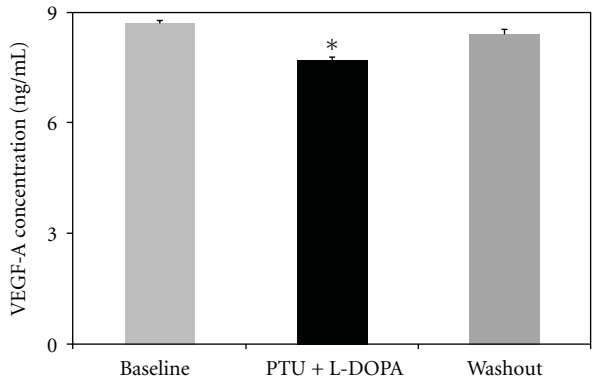

Figure 5: Regulation of VEGF-A expression in hRPE cells. Treatment of hRPE monolayers, grown in 24-well plates, with PTU (a tyrosinase blocker used to eliminate endogenous L-DOPA production) and 1 μM L-DOPA leads to a decrease in VEGF-A concentration in the medium. After washout of PTU and L-DOPA, the VEGF-A concentration returns to the baseline levels. Data are presented as the mean of three experiments conducted in triplicate, error bars represent S.E.M., and an asterisk (*) denotes $P < 0.0005$ using paired t-tests with Bonferroni's correction between the PTU + L-DOPA group from both the baseline and the washout groups.

of hRPE transplantation remains speculative, but is in our opinion deserving of due consideration.

Additional reasons must also be considered to explain the failure of the Phase II clinical trial to show efficacy [13] despite promising preclinical [8–10, 14] and Phase I clinical trials [12]. Subsequent pathology data showed that most cells did not survive implantation for more than 6 months [35], raising doubts about the long-term stability of the graft. Since one of the potential pitfalls of the clinical trial is that the gelatin-based Spheramine microcarriers might have been digested over time, we investigated nondegradable microcarriers in their potential to provide healthy hRPE growth, survival, and neurotrophic output [20, 36]. Culti-Spher microcarriers were ineffective in achieving healthy hRPE monolayers as evidenced by the lack of pigmented cells covering the microcarriers. Thus, it was not surprising that little PEDF or VEGF-A was produced using that substrate. Plastic Plus, ProNectin F, and Hillex II microcarriers on the other hand all have potential as microcarriers for hRPE growth and potential implantation. The hRPE cells appeared the most differentiated, pigmented, and regular in height and thickness on the Hillex II microcarriers. Interestingly, both the PEDF (2–4 ng/mL) and the VEGF-A (15 ng/mL) outputs of the hRPE cells on all three plastic-based microcarriers were very similar throughout the two-month time course, even though the appearance of the cultures was significantly different. We did not investigate the neurotrophic effect of the hRPE cells grown on microcarriers in this study, but based on our prior study evaluating the neurotrophic potential of conditioned media (RPE-CM) from 2-month-old fully differentiated hRPE cultures before and after PEDF depletion [20], we can conclude that over 50% of the neurotrophic effect on cultured neurons that was produced by hRPE cells is due to PEDF production. We can speculate that the remainder of that effect can be mostly attributed to VEGF-A, since we showed in the current study that VEGF-A is secreted throughout the study at significant levels whereas the hRPE cells do not produce measurable amount of BDNF at any time point (data not shown), and the GDNF level (Figure 4) drops below detection threshold by the 3rd week of culture.

In a parallel experiment, we investigated the regulation of the neurotrophic output in hRPE cells by L-DOPA. An interesting facet of the regulation of PEDF secretion is the linkage to an autocrine loop that regulates tyrosinase activity and pigment synthesis utilizing L-DOPA and GPR143. GPR143 is a G-Protein-coupled receptor with highest expression levels found in RPE cells. GPR143 signaling is critical for full expression of the L-DOPA synthetic enzyme tyrosinase. L-DOPA is not only a precursor for the formation of melanin, but also acts to increase melanogenesis by activating the GPR143 receptor creating a positive feedback loop. Loss of GPR143 signaling also reduces the output of PEDF from RPE cells. Genetic defects in GPR143 cause ocular albinism, a disorder of pigmentation that leads to vision loss due to changes in retinal neuron development and survival. The exact cause of the secondary neuronal loss observed in this disorder has been debated, but may reflect the decremental changes in PEDF production and subsequent loss of retinal neurotrophism.

A better understanding of the influence of L-DOPA on the neurotrophic output of RPE cells is also required for the potential use of RPE cell transplantation in PD. Since there is a reciprocal "yin and yang" like effect of PEDF and VEGF-A regulation [17], at work in many cell

types, including the eye, we sought to test whether GPR143 signaling, which upregulates PEDF secretion from hRPE cells, may downregulate VEGF-A secretion. To test this we used the same experimental method used previously to illustrate upregulation of PEDF by GPR143 signaling [21]. VEGF-A secretion was reduced significantly by induction of GPR143 signaling using the PTU/L-DOPA paradigm. Thus, we suggest that in hRPE cells, GPR143 up-regulates PEDF while simultaneously downregulating VEGF-A. In addition to the advance in the field of RPE cell transplantation, this observation has significant implications for our understanding of pigmentation-related eye diseases such as albinism and age-related macular degeneration.

Overall, it is apparent that hRPE cells grown on plastic-based microcarriers produce levels of both PEDF and VEGF-A that are in a range that would support a neuroprotective effect. The levels of PEDF (maximum around 4 ng/mL) were higher than the 1 ng/mL PEDF that was reported to be neuroprotective in both 6-OHDA and rotenone rat midbrain culture models [19]. In this paper we show that the secretion of VEGF-A is downregulated by L-DOPA. This effect was partial (less than 15%) and did not eliminate VEGF-A secretion under any conditions tested. The levels of VEGF-A (maximum around 15 ng/mL) are sufficient to expect a neuroprotective effect, since 1 ng/mL VEGF-A has been shown to provide a maximal protection in a 6-OHDA model of PD [33]. The VEGF-A levels also appear to be low enough that the negative effect of increased vessel formation may not be an issue. In rodent models, pathological vasculogenesis was observed after implantation of BHK cells expressing VEGF-A at approximately 40 ng/mL [33]. It is important to note that future manipulation of the GPR143 pathway could be used to optimize and balance the neurotrophic production of hRPE cells.

In future work the plastic-based hRPE microcarriers should be tested *in vivo* employing rodent models of neurodegeneration, to evaluate the long-term survival of the hRPE and the continued ability to release therapeutically relevant amounts of PEDF and VEGF-A after transplantation into the brain. The relative contribution of PEDF versus VEGF-A to the expected neurotrophic effects should also be further investigated *in vivo*.

5. Conclusion

Human RPE cells can be successfully grown and differentiated on 3 different types of FDA-approved plastic-based microcarriers over an extended period of time. The hRPE microcarriers produce significant amounts of two growth factors with neuroprotective potential, PEDF and VEGF-A. The regulation of both factors can be reciprocally regulated by modulation of the GPR143/OA1 pathway.

Acknowledgments

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