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Assays for the Antiangiogenic and Neurotrophic Serpin Pigment Epithelium-Derived Factor

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Abstract

Pigment epithelium-derived factor (PEDF) is a secreted serpin that exhibits a variety of interesting biological activities. The multifunctional PEDF has neurotrophic and antiangiogenic properties, and acts in retinal differentiation, survival, and maintenance. It is also antitumorigenic and antimetastatic, and has stem cell self-renewal properties. It is widely distributed in the human body and exists in abundance in the eye as a soluble extracellular glycoprotein. Its levels are altered in diseases characterized by retinopathies and angiogenesis. Its mechanisms of neuroprotection and angiogenesis are associated with receptor interactions at cell-surface interfaces and changes in protein expression. This serpin lacks demonstrable serine protease inhibitory activity, but has binding affinity to extracellular matrix components and cell-surface receptors. Here we describe purification protocols, methods to quantify PEDF, and determine interactions with specific molecules, as well as neurotrophic and angiogenesis assays for this multifunctional protein.

1. Introduction

Pigment epithelium-derived factor (PEDF) is an extracellular serpin protein that lacks demonstrable serine protease inhibitory activity, but exhibits a variety of interesting biological properties (Becerra, 1997, 2006; Filleur *et al.*, 2009). It is broadly distributed in the human body. It exists in abundance in the human eye, but its levels are altered in diseases characterized by retinopathies, such as age-related macular degeneration, diabetic retinopathy (Barnstable and Tombran-Tink, 2004; Bouck, 2002). The multifunctional PEDF has neurotrophic and antiangiogenic properties and acts in retinal differentiation, survival, and maintenance. Efficacy was demonstrated (i) in retinoblastoma cells and primary developing motor neurons by promoting neurite-outgrowth; (ii) in primary retinal cells, primary cerebellar granule cell neurons, primary hippocampal neurons, and primary motor neurons by protecting against apoptotic cell death that is associated with toxins and oxidative stress; as well as (iii) in a variety of endothelial cells by promoting proapoptotic mechanisms and preventing cell migration, and by inhibiting endothelial tube formation and vessel sprouting (Tombran-Tink and Barnstable, 2003). PEDF also has antitumorigenic and antimetastatic activities (Broadhead *et al.*, 2009) and it has self-renewal properties on neural stem cell and human embryonic stem cell (Gonzalez *et al.*, 2010; Ramirez-Castillejo *et al.*, 2006).

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. 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. Moreover, PEDF is a potential diagnostic tool for several ocular diseases triggered by pathological neovascularization, retinal degenerations, or tumors. Given the above and that studies on the mechanisms of PEDF action are associated with receptor interactions at cell-surface interfaces and changes in protein expression, there are great interests in methodologies to measure PEDF levels and the interactions of PEDF with components of its natural milieu. Here we describe purification protocols, quantification and binding assays, and bioassays for this multifunctional protein.

2. Purification of PEDF Protein

PEDF is a soluble, extracellular, monomeric glycoprotein of an apparent molecular weight of ~50,000 (Wu *et al.*, 1995). In mammalian eyes, it is abundant in the vitreous (Wu and Becerra, 1996), and is highly concentrated in the interphotoreceptor matrix (IPM; Wu *et al.*, 1995). It is also present in aqueous humor (Ortego *et al.*, 1996), blood (Petersen *et al.*, 2003), cerebrospinal fluid (Kuncl *et al.*, 2002), bone and cartilage (Quan *et al.*, 2005). PEDF amounts ≤1% of total soluble protein of these sources and can be purified from them successfully. Bovine eyes are a good source of PEDF because of their large size, that is, the volume of a bovine vitreous cavity is about three to four times larger than that of human and monkey vitreous and it dislodges easily than that of primate eyes.

2.1. Interphotoreceptor matrix

The soluble components of the IPM can be obtained by the “no-cut” method to assure that the extracellular fluid is free of significant cellular contamination (Adler, 1989). The following is a description of an extraction procedure from bovine eyes that can be adapted to samples from other species:

1. All procedures are to be performed at 4 °C and fresh adult bovine eyes are kept on ice during dissection.
2. 30–50 eyes are dissected at a time. Each eye is dissected as follows: the periocular tissue is trimmed, and the anterior segment and the vitreous are removed leaving an eyecup.
3. A solution of phosphate buffered saline (PBS) is gently introduced between the neural retina and the RPE with a needle at 0.5 ml per eye. The eye is rocked to allow the PBS solution to run through the entire surface.
4. The PBS solution is extracted by aspiration, avoiding breakage of the retina membrane, and is transferred to a 50-ml tube.
5. The pooled washes are subjected to centrifugation at 1500×*g* for 15 min to remove cellular debris.
6. The supernatant is filtered through a 0.45-μm syringe filter and stored at −80 °C. This filtrate is termed IPM wash.

2.2. Vitreous humor

1. After trimming the periocular tissue, remove the anterior segment and carefully peel the vitreous body from the neural retina.
2. The vitreous is collected at approximately 10–12 ml per eye and is placed in a 50-ml tube.
3. Homogenize vitreous gel using a Brinkman Polytron, model PT-MR 3000 (Kinematica AG, Littau, Switzerland) three times at 9500 rpm for 3 s.

4. The liquefied homogenates are subjected to centrifugation at 1300×*g* for 15 min to remove cellular debris. The supernatant is ready to use or store at −80 °C until use.

2.3. Aqueous humor

1. Extract aqueous humor from eyes by keratocentesis and by aspirating the humor with a syringe connected to a 25-gauge needle.
2. The pooled aqueous humor extracts are subjected to centrifugation at 1300 × *g* for 15 min to remove cellular debris. The volume of a humor per bovine eye is approximately 1.5 ml. The supernatant is ready to use or store at −80 °C until use.

2.4. Plasma

1. Blood is collected using blood collection tubes with heparin (BD Vacu-tainer, BD Diagnostics, Oxford, UK), and centrifuged at 1000×*g* for 15 min at room temperature. The serum can be used immediately or stored at −80 °C.
2. Serum samples are albumin-depleted using the Qproteome Murine Albumin Depletion Kit following manufacturer's instructions (Qiagen, Valencia, CA, USA) before use. This step removes the most abundant protein in serum and allows a more precise analysis of low-abundant proteins, such as PEDF.

2.5. Recombinant PEDF

Heterologous expression of PEDF from several species can be achieved using prokaryotic or mammalian cells. *Escherichia coli* cells expressing PEDF can be used as starting material for purification (Becerra *et al.*, 1993). Stably transfected mammalian cells (e.g., BHK, HEK-293 cells) with expression vectors containing full-length PEDF cDNA produce and secrete PEDF to the conditioned media (Duh *et al.*, 2002; Perez-Mediavilla *et al.*, 1998; Sanchez-Sanchez *et al.*, 2008; Stratikos *et al.*, 1996). Transfected cells are cultured to confluency in roller bottles with the complete culturing media for 24 h and then without serum for 24 h in repetitive cycles. After each cycle, media without serum is harvested, filtered, and stored at −80 °C or used immediately as starting material for biochemical fractionation.

2.6. Biochemical fractionation

Starting with an IPM wash or vitreous sample, PEDF protein can be purified >150-fold to near homogeneity by ammonium sulfate fractionation and cation-exchange chromatography, with a recovery of >40% (Wu *et al.*, 1995). The PEDF from bovine extracts remains in suspension at 45% ammonium sulfate saturation, and can be used as a first purification step. For PEDF from other sources, 80% ammonium sulfate precipitation can be used as a protein concentration step. Highly purified recombinant PEDF protein has been obtained at milligram amount per liter of cell culture (Stratikos *et al.*, 1996).

2.6.1. Ammonium sulfate fractionation

1. A total of 258 mg ammonium sulfate is added per 1 ml extract (above).
2. The suspension is stirred for 2 h and then centrifuged at 40,000×*g* for 2 h.
3. The supernatant fraction (S_{45}) is mixed with an additional 226 mg ammonium sulfate per milliliter to achieve 80% saturation, and stirred and fractionated as in step 2.

4. The precipitated fraction (P_{80}) is resuspended thoroughly in PBS solution; for example, use 1 ml PBS to resuspend P_{80} from IPM from every 25 eyes or from vitreous from 1.6 eyes.
5. The suspension is dialyzed against buffer S (50 m*M* sodium phosphate, pH 6.4, 1 m*M* DTT, 10% glycerol) containing 50 m*M* NaCl for 2 h each of three changes of buffer.
6. The dialyzate is centrifuged and passed through a filter (0.45 μm) to remove particulate material.

2.6.2. Cation-exchange column chromatography—A variety of cation-exchange resins and column sizes can be used, for example, S-Sepharose Fast Flow (Pharmacia) at 1 ml-bed volume for IPM dialyzate from 80 eyes, or vitreal dialyzate from 16 eyes; Mono-S HR5/5 column (10 cm × 1 cm, Pharmacia) for IPM from >500 eyes, or vitreous from more than 100 eyes; or 1.67 ml-bed volume POROS S (Applied BioSystems). The columns can be attached to standard automated fast protein liquid chromatography (FPLC) or perfusion chromatography systems. After equilibration with buffer S containing 50 m*M* NaCl, the samples are loaded on the columns. The unbound material is washed with the same buffer and the bound material is eluted with a linear gradient from 50 to 500 m*M* NaCl in buffer S at a flow rate 0.8–3 ml/min. PEDF elutes at about 200 ± 50 m*M* NaCl.

2.6.3. Anion-exchange column chromatography—The PEDF-containing fractions from the cation-exchange column chromatography are pooled and concentrated, for example, by ultrafiltration with Centricon-30 (Amicon, Beverly, MA). The filtrate is desalted, for example, by ultrafiltration or dialysis against buffer Q (50 m*M* Tris, pH 8.2) containing 50 m*M* NaCl. A variety of resins and column sizes may be used, for example, Q-Sepharose Fast Flow (Pharmacia), POROS Q (Applied BioSystems). The columns can be attached to standard automated FPLC or perfusion chromatography systems. After equilibration with buffer Q containing 50 m*M* NaCl, the samples are loaded on the columns. The unbound material is washed with the same buffer and the bound material is eluted with a linear gradient from 50 to 500 m*M* NaCl in buffer Q at a flow rate 0.8–3 ml/min. PEDF elutes at about 200 ± 50 m*M* NaCl. The PEDF-containing fractions are pooled and concentrated and stored at −80 °C.

3. Techniques to Assay PEDF

3.1. Immunochemical assays

Polyclonal and monoclonal antibodies to PEDF are available from commercial sources. Purified native and recombinant full-length PEDF can be detected as a ~50,000-MW protein in Western blots. Sensitivity of detection of the PEDF-immunoreactive signal is very similar between the colorimetric and chemiluminescent detection methods after Western blotting.

3.1.1. Immunoblot reaction with polyclonal antibody Ab-rPEDF

Solutions and reagents

- **a.** TBST (20 m*M* Tris–Cl, pH 7.5, 150 m*M* NaCl, 0.05% Tween 20)
- **b.** 1% BSA in TBST (filter after dissolving, store at 4°C)
- **c.** Rabbit polyclonal AbPEDF antibody (e.g., BioProducts MD, LLC) or monoclonal anti-PEDF (e.g., Millipore, MAB1059, clone 10F12.2)
- **d.** Biotinylated antirabbit IgG (H + L) [affinity purified antibody made in goat, human serum absorbed KPL cat. No. 16-15-16] (in 50% glycerol, store at 4°C); or

biotinylated anti-mouse IgG (H + L) (affinity purified antibody made in goat, human serum absorbed KPL cat. No. 16-15-16) in 50% glycerol, store at 4 °C

e. ABC: Vectastin ABC elite kit (Vector labs, Inc cat. No. PK-6100) store at 4 °C

f. TBS (20 m*M* Tris–Cl, pH 7.5, 150 m*M* NaCl)

g. HRP color development reagent (Bio-Rad cat. No. 170-6534) stored at −20 °C

1 Incubate blot in 1% BSA/TBST for 1 h at room temperature—or 16 h at 4 °C—with gentle rocking (about 15–20 ml per membrane of 8 cm × 7 cm)

2 Incubate blot in polyclonal or monoclonal AbPEDF 1:1000–1:4000 in 1% BSA/TBST for 1 h at room temperature or more diluted than 1:4000 at 4 °C for 16–24 h with gentle rocking (about 10 ml per membrane of 8 cm × 7 cm). (*Note*: the diluted antiserum may be reused later, save if necessary, store at 4 °C. Its titer might decrease with time and use, whatever comes first.)

3 Wash membrane with 25 ml of TBST, rock at room temperature for 5 min, three times

4 Incubate blot in biotinylated anti-rabbit IgG (H + L) or anti-mouse IgG (H + L) 1:1000 in 1% BSA/TBST, with gentle rocking for 1 h at room temperature (about 10 ml per membrane 8 cm × 7 cm). (Prepare ABC, see step 6.)

5 Wash membrane with 25 ml of TBST, rocking at room temperature for 5 min each wash, three times

6 Incubate blot in ABC for 30 min at room temperature

Note: ABC must be prepared in advanced!

ABC: Vectastin ABC elite kit:

to 10 ml TBST add three drops Solution A, mix by inversion then add three drops Solution B, mix by inversion let ABC stand 30 min at room temperature before using

7 Wash membrane with 25 ml of TBS, rock at room temperature for 5 min each wash, three times

8 Develop color with freshly prepared solution D

Solution D

- dissolve 12 mg of HRP color development reagent in 4 ml methanol
- add 20 ml TBS, mix
- mix in 12:l 30% H_2O_2
- let color develop up to 30 min, if necessary

9 Rinse blot with water to stop development reaction and air dry. Store membrane between pieces of 3 MM paper and cover with aluminum wrap

3.1.2. ELISA—The best way to quantify PEDF in heterologous samples is by ELISA. Several ELISA kits are available in the market. We have used one by BioProducts MD because of its high sensitivity and it specifically detects PEDF. The following link is for step-by-step instructions on its use http://www.bioproductsmd.com/Documents/PEDF_ELISA_Manual.pdf

3.2. Binding activities

PEDF has affinity for extracellular matrix components such as glycosami-noglycans and collagens (Alberdi *et al.*, 1998; Becerra *et al.*, 2008; Meyer *et al.*, 2002). The following procedures are for assaying binding to heparin, heparin sulfate, hyaluronan, and collagen.

3.2.1. Glycosaminoglycans

3.2.1.1. Glycosaminoglycan-affinity column chromatography: Heparin-or hyaluronan-affinity resins can be prepared as described earlier (Alberdi *et al.*, 1998; Becerra *et al.*, 2008) or from commercial sources (e.g., Sigma). Optimum PEDF binding is obtained in phosphate buffers containing NaCl concentrations below 100 m*M* and at pH values between 6 and 7.

1. Affinity resins are packed in Polyprep chromatography columns (Bio-Rad) to yield 0.5 ml settled bed volume and equilibrated with buffer H (20 m*M* NaCl, 20 m*M* sodium phosphate, pH 6.5, and 10% glycerol).
2. A solution of protein (PEDF up to 40 μg) in buffer H is applied to the appropriate affinity column and incubated with the resin at 4 °C for 30 min.
3. The glycosaminoglycan-affinity columns are washed with more than 10 column volumes of the incubation buffer.
4. Elution is with an NaCl step-gradient in buffer H at 1.5 column volumes per fraction.

3.2.1.2. Cetylpyridinium chloride precipitation: Hyaluronan binding in solution can be assayed by precipitation with cetylpyridinium chloride (CPC). Optimum PEDF binding to hyaluronan occurs in buffers with pH 7.5–8.0 and with NaCl concentrations ≤300 m*M* (Becerra *et al.*, 2008).

1. Solutions of 10–100 μg/ml PEDF and 10–1000 μg/ml hyaluronan are mixed in PBS containing BSA as carrier (e.g., 250 μg/ml) and incubated at 4 °C for 60 min.
2. Add 1× volume of 2.5% CPC in PBS and incubate at 37 °C for 1 h.
3. Separate the precipitate by centrifugation in an Eppendorf centrifuge at 16,000×*g* for 15 min at 4 °C.
4. Discard the supernatant and wash the precipitate with 200 μl of 1% CPC in PBS.
5. Resuspend the precipitate in 25–30 μl of sample buffer for SDS-PAGE.
6. Protein detection in gels is performed with Coomassie Blue staining or immunostaining after Western blotting with antibodies to PEDF.

3.2.2. Heparan sulfate proteoglycan—These assays were based on the separation of complexes formed between PEDF and other proteins by ultrafiltration in which PEDF complexes >100-kDa are retained by a membrane of M_r 100,000 exclusion limit, while free PEDF molecules of 50 kDa are filtered through.

3.2.2.1. Radioactive free assay

1. PEDF (120 μg/ml) is mixed with Heparan sulfate proteoglycan (HSPG; 50–300 μg/ml) in buffer H in a final volume of 100 μl.
2. The mixtures are incubated at 4 °C for 30 min and then ultrafiltered through membranes with a molecular cut-off >100,000 (e.g., Centri-con-100 devices, Millipore).

3. The concentrated material is diluted 20-fold with incubation buffer and ultrafiltered. This is repeated four times.
4. Aliquots of the concentrated samples are analyzed by SDS-PAGE.

3.2.2.2. Radioactivity binding assay

1. Radiolabeled [^{125}I]PEDF at 90 ng/ml (6.25 μCi/ml) and increasing concentrations of HSPG are mixed in buffer H (10 μl) and incubated at 4 °C for 30 min.
2. Free and bound PEDF are separated by ultrafiltration through Microcon-100 (Amicon). The concentrated material is diluted 40-fold with incubation buffer and washed as described above.
3. Each Microcon retenate cup is transferred to a scintillation vial and mixed with 5 ml of Bio-Safe II liquid scintillation solution (Research Products International, Corp.) by extensive vortexing. Radioactivity is determined using a β-counter (Beckman, model LS 3801).

3.2.3. Collagen

3.2.3.1. Solution binding assays: PEDF proteins (100 μg/ml) are mixed with collagen (100 μg/ml) in PBS, pH 7.4, containing 10% glycerol and incubated at 4 °C for 1 h. If stock solutions of collagen contain acetic acid, NaOH can be added to neutralize the pH of the reaction mixtures. PEDF complexes are separated from free PEDF by ultrafiltration as described above for HSPG using Centricon-100 devices. Bound PEDF can be analyzed in the retained material by SDS-PAGE and visualized by Coomassie Blue staining or immunostaining, and it can be quantified by ELISA.

3.2.3.2. Solid-phase binding assays

1. Binding reactions are performed with ^{125}I-PEDF (e.g., 2 n*M*) and increasing amounts of unlabeled PEDF in 0.1% BSA/PBS, pH 7.4, to collagen I immobilized on plastic of 24-well plates.
2. After incubations at 4 °C for 90 min with gentle rocking, the binding solution is removed, and the wells are washed three times with 0.1% BSA/PBS.
3. Then 1 N NaOH is added to the wells, incubated at room temperature for 30 min, and transferred to scintillation vials to determine the amount of radioactivity using a β-scintillation counter.
4. Nonspecific binding is determined from fractions with >100-fold molar excess of unlabeled PEDF over radioligand.
5. Binding data can be analyzed by nonlinear regression using GraphPad Prism software.

3.2.3.3. Surface plasmon resonance assays: Assays for PEDF-collagen I interactions can be performed immobilizing either 4 ng of collagen I or PEDF on a CM5 sensor chip, by *N*-hydroxysuccinimide (NHS)/EDC activation, followed by covalent amine coupling of the proteins to the surface using Biacore 3000. Treat the carboxymethyl-dextran surface of the sensor chip with NHS/EDC to activate it in preparation for amine coupling. The NHS/EDC creates reactive ester groups where the carboxyl groups were on the dextran (only about 40% of the COO^- groups are derivatized). The protein designed to be bound to the activated CM5 chip is exposed to the dextran with the reactive esters. Primary amines on the protein (e.g., N-terminal and possibly lysine groups) then attach to the dextran by nucleophilic substitution of the reactive ester. The remaining free surface (about 60%) is then blocked

with 0.1 *M* Tris, pH 8.0, and the matrix washed with 0.5 *M* NaCl solution and then reequilibrated with binding buffer (PBS, 10% glycerol). Eight different dilutions of PEDF or collagen I are prepared in binding buffer with concentrations ranging from 0 to 1.0 μM and injected from low to high concentration, and then the series is repeated, to study the interaction of both free PEDF on a collagen I matrix and the inverse orientation. Each injection is followed by a 0.5 *M* NaCl regeneration step. The data are then fitted to several binding models for a kinetic analysis. The best fittings are obtained with a simple 1:1 Langmuir model for the collagen surface binding assay and with a bivalent analyte model for the opposite orientation.

3.2.4. PEDF receptor proteins

3.2.4.1. Radiolabeled ^{125}I-PEDF binding assays

3.2.4.1.1. Cells in suspension

1. Cells in suspension ($6 \times l0^5$ cells/ml) are incubated at 4 °C for 15 min before the addition of ^{125}I-PEDF (0.1–2 n*M*). *Note*: binding is enhanced when binding buffer is media conditioned overnight without serum (Alberdi *et al.*, 2003).
2. The binding reaction mixture is incubated at 4 °C for a period of time between 15 and 90 min.
3. The reaction is terminated by the addition of 10 ml of ice-cold PBS supplemented with 0.1% BSA.
4. Immediately the reaction is subjected to filtration under vacuum through Whatman GF/C filters presoaked in 0.3% polyethylenimine.
5. Finally, the filters are washed with 10 ml of the ice-cold 1% BSA in PBS.

3.2.4.1.2. Attached cells

1. Attached cells are cultured in 24-well plates, to 90% confluency or containing 5×10^5 cells/well.
2. Cells are washed with 0.5 ml of 0.1% BSA in culturing medium (binding buffer) three times before the addition of ^{125}I-PEDF in binding buffer.
3. After incubation at 4 °C for 15–90 min, the unbound PEDF is washed with 0.5 ml of binding buffer three times.
4. Then the cells are lysed by incubation with 0.5 ml of 1 *M* NaOH at room temperature for 30 min.

3.2.4.1.3. Determination of bound PEDF: Filters and cell lysates are placed in scintillation vials, mixed with 5 ml of Bio-Safe II liquid scintillation solution (Research Products International, Corp.), incubated at room temperature overnight, and then mixed by extensive vortexing before determining the radioactivity using a β-counter (Beckman, model LS 3801). Alternatively, bound and free radioligand are separated by centrifugation of cell suspensions followed by three washes with 1% BSA in PBS, and the bound radioactivity is determined in the cell pellets using a gamma counter (Wallac) or after SDS-PAGE and autoradiography. Nonspecific binding is defined as the amount of bound radioactivity in the presence of saturating concentrations of unlabeled ligand, and specific binding as bound radioactivity minus nonspecific binding. Data are analyzed using the Minitab statistical program and Microsoft Excel for linear regression, as well as GraphPad Prism for nonlinear regression and Scatchard analyses.

3.2.4.2. Ligand-affinity column chromatography

1. Fresh detergent-soluble membrane fractions from tissues or cell in culture are prepared as described (Alberdi *et al.*, 1999; Aymerich *et al.*, 2001).
2. Highly purified PEDF protein is coupled to beads of preactivated hydrophilic, cross-linked bis-acrylamide/azlactone copolymers (3M Emphaze Ultralink; Pierce, Rockford, IL).
3. Detergent-soluble membrane proteins (0.1–1 mg) are passed through a column of resin without ligand (2 ml).
4. The unbound material is mixed with PEDF-coupled resin (2 ml; ~6 mg PEDF/ml resin) and gently rotated at 4 °C for 1 h.
5. The material is packed in a column, washed with buffer D (20 column volumes or until absorbance at 280 nm was undetectable), followed by 1*M* NaCl in buffer D (10 column volumes).
6. The bound material is eluted with 0.1 *M* glycine buffer, pH 11, 10% glycerol, 1 m*M* $CaCl_2$, 0.15 NaCl, and 0.25% CHAPS (10 column volumes).
7. Eluted proteins are concentrated to 100 μl by ultrafiltration with micro-concentrators (Centricon-30, Millipore).

3.2.4.3. Ligand blot

1. Detergent-soluble membrane proteins are resolved by SDS-PAGE under nonreducing conditions and transferred to a 0.2-μm nitrocellulose membrane.
2. The membrane is first washed with 1% NP-40 in TBS for 15 min and then twice with TBS at 25 °C for 10 min each.
3. The blot is incubated with blocking solution (1% BSA in TBST, containing TBS with 0.05% Tween 20) at 25 °C for 2 h.
4. The blot is incubated with ^{125}I-PEDF (2 n*M*) in blocking solution at 4°C for 16 h.
5. The blot is washed three times with TBST at 25 °C for 15 min to remove the unbound ligand.
6. The blot is air dried, and exposed to X-ray film (BioMax ML, Eastman Kodak Co., Rochester, NY) to detect bound radioligand by autoradiography.

4. Neurotrophic Assays

PEDF acts on the retina *in vivo* and on live cells. It supports normal development of photoreceptor neurons (Jablonski *et al.*, 2000). In addition, it can delay the death of photoreceptors in mouse models of inherited retinal degenerations (Cayouette *et al.*, 1999), and it can protect photorecepotors from light-induced damage (Cao *et al.*, 2001). It can protect the neural retina against ischemic injury (Takita *et al.*, 2003). Moreover, PEDF can induce morphological differentiation of retinoblastoma cells into a neuronal phenotype. It can promote neurite-outgrowth on Y-79 and Weri cells as well as in spinal cord motor neurons (Becerra, 1997; Houenou *et al.*, 1999). It can protect mixed retina cells, retinal ganglion cells, retinal pigment epithelial cells, and developing hippocampal neurons from death by several insults (DeCoster *et al.*, 1999; Notari *et al.*, 2005; Pang *et al.*, 2007; Tsao *et al.*, 2006).

4.1. Neurite-outgrowth analyses

1. Human Y-79 retinoblastoma cells (ATCC) are cultured in suspension in MEM supplemented with 15% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 pg/ml streptomycin) at 37 °C in a humidified incubator under 5% CO_2.
2. Cells are propagated for two passages after receipt and then frozen in the same MEM medium containing 10% dimethyl sulfoxide. Separate aliquots of cells are then used for each differentiation experiment.
3. After thawing, cells are kept in suspension culture without further passaging in serum-containing MEM until the appropriate number of cells is available.
4. Cells are collected by centrifugation, washed twice, and resuspended in PBS and counted.
5. For treatment with PEDF, 2.5×10^5 cells are seeded into each well in 6-well plates (Nunc, Inc.) with 2 ml of serum-free medium consisting of MEM supplemented with 1 m*M* sodium pyruvate, 10 m*M* HEPES, 1× nonessential amino acids, 1 m*M* L-glutamine, and 0.1% ITS mix and antibiotics as above.
6. Approximately 12–16 h later, PEDF (50–200 ng/ml) is added to the medium.
7. The cultures are incubated and kept undisturbed for 7 days. Cells under these conditions remain in suspension.
8. On the 8th day after treatment, cells are transferred to 6-well plates precoated with poly-D-lysine (Collaborative Research); once the cells attach to the substrate (about 6–8 h), the old medium is replaced with 2 ml of fresh serum-free medium. The cultures are maintained under these conditions for up to 11 days.
9. Using an Olympus CK2 phase-contrast microscope, postattachment cultures are examined daily for morphological differentiation and quantification of neurite-outgrowth.

4.2. Retina cell survival assays

PEDF can protect cell death induced by serum starvation in a rat retinal precursor R28 cell line (Notari *et al.*, 2005). The R28 cell line is derived from postnatal day 6 Sprague–Dawley rat retina, immortalized with the 12S E1A gene of adenovirus using incompetent retroviral vector (Seigel *et al.*, 1996). These cells are provided by Dr. Gail Siegel (SUNY, Buffalo, NY). The survival activity assay for PEDF is performed as follows using cells with passage numbers 45–55:

1. R28 cells are cultured in Dulbecco's modified Eagle's medium (DMEM) incomplete media with 3% sodium bicarbonate, 1× MEM nonessential amino acids, 1× MEM vitamins, 2 m*M* L-glutamine, gentamicin 0.1 mg/ml, 10% of bovine calf serum, and 1% of Penicillin/Streptomycin (P/S) at 37 °C with 5% CO_2. Cells are propagated maintaining them at not more than 70% confluency and are detached from the flask using 5 m*M* EDTA in 1× PBS.
2. Cells are seeded at a density of 2×10^4 cells/well in 24-well plates in media containing 5% of bovine calf serum, and allowed to attach for 8 h.
3. At the end of 8 h, media is removed, cells are washed once with 1× PBS, and serum-free media or serum-free media containing human recombinant PEDF at desired concentrations is added to each well.
4. Cell viability is measured at the end of 48 h, for example, using the CellTiter-Glo™ viability assay kit (Promega). This kit uses a unique, stable form of

luciferase to measure ATP as an indicator of viable cells and the luminescent signal produced is proportional to the number of viable cells present in culture. Briefly, cells are washed once with 1× PBS. This is followed by addition of 100 μl of PBS and 100 μl of Cell-Glo reagent (thawed to room temperature) to each well. The plate is incubated for 10 min at room temperature and luminescence signal can be measured using Envision automated plate reader (Perkin Elmer, MA).

4.3. Protection against oxidative damage

PEDF protects retinal pigment epithelium from apoptosis induced by oxidative stress as demonstrated earlier (Mukherjee *et al.*, 2007). The assay is performed as follows:

1. ARPE-19 cells (ATCC) are cultured in DMEM/F-12 (1:1), penicillin/ streptomycin, 0.5 mg/ml Geneticin, and 10% fetal bovine serum at 37°C, 5% CO_2.
2. Cells are trypsinized and plated at a density of 1×10^5 cells/well in 24-well plates. The cells are allowed to grow to 100% confluency (72 h) which is very critical for the assay.
3. Media is removed and media containing 0.5% serum is added to starve the cells for 8 h. Then the cells are treated with 30% H_2O_2 to achieve the desired final concentrations and TNF α (10 ng/ml). The optimal H_2O_2 concentration for oxidative damage can be determined in preliminary experiments using concentration ranges (400–800 μ*M*). PEDF (10 ng/ml) along with DHA (30 n*M*) are added along with H_2O_2 and TNF-α.
4. Cells are incubated for 16 h and fixed with methanol for 15 min at room temperature.
5. Cells are then washed with 1× PBS followed by addition of 5 μ*M* Hoechst reagent in PBS for 15 min at room temperature.
6. Cells are washed once with 1× PBS and different fields are imaged using UV fluorescence under a Nikon Eclipse TE2000-U microscope.
7. The percentage of pyknotic cell nuclei as seen by condensed morphology in the field is counted from the digital images.

5. Antiangiogenic Assays

5.1. Chick embryo aortic arch assay

The chick embryo aortic arch assay is an *ex vivo* angiogenesis assay as previously described (Martinez *et al.*, 2004).

1. Aortic rings of approximately 0.8 mm in length are prepared from the five aortic arches of 13-day-old chicken embryos (CBT Farms, Chestertown, MD).
2. The soft connective tissue of the adventitia layer is carefully removed with tweezers.
3. Each aortic ring is placed in the center of a well in a 48-well plate and covered with 10 μl of synthetic matrix (Matrigel; BD Biosciences, San Jose, CA).
4. After the matrix solidified, 300 μl of growth-factor–free human endothelial serum-free basal growth medium (Invitrogen) containing the proper concentration of the test substances is added to each well.
5. The plates are kept in a humid incubator at 37 °C in 5% CO_2 for 24–36 h.

6. Microvessels sprouting from each aortic ring are photographed in an inverted microscope and the area covered by the newly formed capillaries is estimated.
7. Endothelial cell growth supplement (ECGS; Biomedical Collaborative Products, Bedford, MA) is used at 400 μg/ml as an angiogenesis promoter. Six independent rings per treatment are measured as replicates.

5.2. Directed *in vivo* angiogenesis assay

Analysis and quantitation of angiogenesis is done using a directed *in vivo* angiogenesis assay (DIVAA) as previously described (Martinez *et al.*, 2002).

1. Ten millimeter long, surgical-grade silicone tubes with only one end open (angioreactors) are filled with 20 μl of synthetic matrix alone or mixed with VEGF and/or rhuPEDF.
2. After the matrix solidified, the angioreactors are implanted subcutaneously into the dorsal flanks of anesthetized athymic nude mice (National Cancer Institute [NCI] colony).
3. After 11 days, the mice are injected intravenously (IV) with 25 mg/ml FITC-dextran (100 μl/mouse; Sigma-Aldrich) 20 min before the angioreactors are removed.
4. Quantitation of neovascularization in the angioreactors is determined as the amount of fluorescence trapped in the implants and is measured in a spectrophotometer (HP; Perkin Elmer Life Sciences). Eight implants are used per treatment point as replicates.

This protocol was approved by the internal NIH animal committee and was in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

5.3. Cell migration

This assay was modified from a protocol previously described (Polverini *et al.*, 1991):

1. Prepare gelatinized 0.5 or 0.8 μm Nuclepore membranes as follows: incubate overnight in 0.5 *M* glacial acetic acid at room temperature with *gentle* shaking, wash three times for 1 h each in autoclaved milli-Q H_2O (mQH_2O) at room temperature with gentle shaking, incubate overnight in 0.01% gelatin in sterile PBS (Ca^{2+} and Mg^{2+} free); Difco gelatin (cat. No. 214340) for at least 4 h to O/N at room temperature (I prefer O/N); rinse in sterile mQH_2O for 5 min and air dry 1 h. Store membranes between pieces of whatmann paper or between the papers that come with the membranes and use within ~1–2 months.
2. Rinse a confluent T75 flask of microvascular endothelial cells with PBS, and replace with basal media + 0.1% BSA to starve cells. Incubate at least 4 h to O/N (16 h, maximum).
3. Harvest the cells by trypsinization, resuspending at a cell concentration of 1.0–1.5 × 10^6 cells/ml in basal media + 0.1% BSA.
4. Rinse Boyden chamber wells with 1× PBS (Ca^{2+} and Mg^{2+} free), 29 μl/well and flick chamber to remove PBS.
5. Add 29 μl of the cell suspension per well in Boyden chamber, remixing cells after every 3rd to 4th well by pipetting up and down.

6. Using forceps, carefully place membrane over cells, *shiny side up* and avoiding air bubbles between the cells and membrane. Assemble chamber per the manufacturer's instructions.
7. Invert Boyden chamber, wrap loosely in aluminum foil (to prevent evaporation), and incubate 1.5–2 h at 37 °C, 5% CO_2 to allow cells to attach to the lower side of the membrane.
8. Prepare 210 μl of test samples and controls in media + 0.1% BSA. The media + 0.1% BSA serves as the negative control, and VEGF (100–1000 pg/ml) or bFGF2 (10–50 ng/ml) in media + 0.1% BSA, positive control. Test PEDF for inhibition against positive control or test substance at 1–10 n*M*. For other test samples, an initial dilution curve may be required to determine optimal dose.
9. Load 51–52 μl per well in quadruplicate for each control and test sample and incubate as above for 3–4 h, loosely wrapped in foil.
10. Dismantle the chamber as per the manufacturer's instructions and notch membrane to maintain orientation.
11. Fix the cells by 1-min incubation in fixative, then stain the membrane for one to two each in solution 1 (orange) and then solution 2 (purple), rinse briefly in PBS and air dry on paper towels for 1–3 h or O/N, shiny side up (be sure membrane does not stick to paper).
12. Cut the membrane in half, notching the second half in a manner to maintain orientation, and mount on glass slide, shiny side up, using a convenient mounting medium (Cytoseal XYL), then coverslip.
13. Count cells that have migrated to the upper side of the membrane which is the side in which the pores of the membrane are in focus. The majority of cells should be on the side of the membrane in which the pores are not in focus. Count 10 randomly chosen high powered fields for each replicate of each sample well at 100× (oil immersion). Choose a field by scanning the lower side of the membrane (unmigrated cells), then refocus to the upper side to count migrated cells to avoid field bias.

5.4. Choroidal neovascularization

PEDF and PEDF-derived peptides can attenuate choroidal neovascularization (CNV) induced with laser (Amaral and Becerra, 2010). The following method has been described earlier (Campos *et al.*, 2006).

5.4.1. Laser-induced CNV

1. Brown Norway male rats (Charles River Laboratories, Rockville, MD) weighing between 300 and 350 g are used.
2. Rats are anesthetized with an intraperitoneal injection of a 40–80 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 10–12 mg/ kg xylazine (Ben Venue Laboratories, Bedford, OH) mixture.
3. Topical 0.5% proparacaine is applied and pupils are dilated with a mixture of 1% tropicamide and 2.5% phenylephrine (Alcon Fort Worth, TX).
4. Hot pads maintain the body temperature while rats are placed in front of a slit lamp.
5. Four to eight shots surrounding the optic nerve are placed with an ND: YAG 532-nm laser (Alcon) using a 5.4-mm contact fundus laser lens (Ocular Instruments,

Bellevue, WA), a spot size of 50 μm, power between 80 and 90 mW, and 0.100 s of exposure time. The end point "bubble formation" assures breakage of Bruch's membrane (Dobi *et al.*, 1989).

6. The animals are euthanized by CO_2 exposure after specific periods of time after injury.

This protocol was approved by the internal NIH animal committee and was in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

5.4.2. Flatmount technique

1. With the nictitans membrane (nasal) used for orientation, eyes are enucleated and immediately fixed in 4% paraformaldehyde (EM Grade; Polysciences, Inc. Warrington, PA) in PBS (9 g/l NaCl, 0.232 g/l KH_2PO_4, 0.703 g/l Na_2HPO_4, pH 7.3) for 1 h.
2. Under a dissecting microscope, the anterior segment, and crystalline lens are removed, and the retinas are detached and separated from the optic nerve head with fine curved scissors.
3. The remaining eye cups are washed with cold ICC buffer (0.5% BSA, 0.2% Tween 20, 0.05% sodium azide) in PBS.
4. A 1:1000 dilution of a 10-mg solution of 4′,6-diamidino-2-phenylin-dole (DAPI), a 1:100 dilution of a 1-μg/μl solution of isolectin IB_4 conjugated with Alexa Fluor 568, and a 1:100 dilution of a 0.2 units/μl solution of phalloidin conjugated with Alexa Fluor 488 (Invitrogen-Molecular Probes, Eugene, OR) are prepared in ICC buffer and centrifuged for 1 min at 2040 × *g*.
5. Alternatively, CD11b (MCA275R; Serotec, Oxford, UK) conjugated with Alexa Fluor 488, an antibody that labels microglia in retina and brain, is used at dilutions of 1:200 to identify retinal microglia.
6. A humidified chamber is prepared, the eye cups are covered with fluorescent dyes prepared as described earlier, incubated at 4 °C with gentle rotation for 4 h, and washed with cold ICC buffer.
7. Radial cuts are made toward the optic nerve head, and the sclera-choroid/RPE complexes are flatmounted (Gel-mount; Biomedia Corp. Foster City, CA), covered, and sealed.

5.4.3. Subconjunctival injections—Protein injections can begin immediately after laser injury and be repeated daily until day 4. Doses of 10, 1, 0.1, and 0.01 pmol PEDF, or PEDF peptides per injection have been used. Lasered eyes with no injection, PBS injection, and angiostatin are used as negative and positive controls. The animals are euthanized by CO_2 exposure at day 7. The effect of PEDF after CNV has been induced can be tested. In another set of experiments, daily PEDF injections can start at the 7th day after laser and continue until day 11 after laser. The animals are euthanized by CO_2 exposure at day 14 after laser.

1. Protein or peptide stock solutions are diluted in PBS and filtered sterilized. Dilutions are prepared so that 2 μl contain the desired dosage per animal.
2. Two microliters are injected into the subconjunctiva of each eye while the animal under general anesthesia for restraint. Samples are administered daily.

5.5. Corneal pocket

1. Prepare Hydron pellets in sterile hood. Cut nylon mesh (Spectrum, cat. No. 148391) into rectangles roughly 3 cm × 4 cm and sterilize by soaking in 70% ethanol for 30 min and allow to air dry. Suspend 5 mg Sucralfate (Bukh, MediTec, cat. No. 95092601) in 10 μl test substance in PBS. For positive control, use 5 μl of 250 ng/ml FGF2. Test PEDF at 8 n*M*. Add 10 μl 12% Hydron (Interferon Sciences, New Brunswick, NJ) prepared at least 10 h in advance in 96% EtOH. Resuspend well.
2. Spread resulting solution on mesh area of approximately 10 × 5 squares of mesh. Repeat several times if necessary. Allow 10–15 min for gel to polymerize. The pellets will be embedded in the mesh. Spread additional 5 μl of Hydron solution on both sides of the embedded pellet area. Allow another 5–10 min to dry. Carefully remove threads of the mesh in one dimension, releasing the pellets (watch for static electricity).
3. Anesthetize female Fischer 344 rats (Harlan Industries, Indianapolis, IN) weighing 120–140 g, and rinse the eye with several drops of proparacaine, blot. Assay can be performed in mice as well.
4. Make an incision across the center of the eye with #15 surgical blade. Using a modified iris spatula, create a pocket. Insert a pellet with fine forceps, and seal the incision with ophthalmic antibiotic ointment.
5. Briefly, Hydron pellets (Interferon Sciences) of <5 μm were prepared containing the test sample CM or bFGF (0.15 μ*M*, positive control). Pellets were implanted into the avascular corneas of anesthetized rats 1.0–1.5 mm from the limbus.
6. At 7 days postimplantation, perfuse animal with colloidal carbon to visualize the vascularity and for a permanent record of the response.

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