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Localization of pigment epithelium derived factor (PEDF) in developing and adult human ocular tissues

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Purpose: To localize pigment epithelium-derived factor (PEDF) in developing and adult human ocular tissues.
Methods: PEDF was localized in fetal and adult eyes by immunofluorescence with a polyclonal antibody (pAb) against amino acids 327-343 of PEDF, or a monoclonal antibody (mAb) against the C-terminal 155 amino acids of PEDF. Specificity of the antibodies was documented by Western blotting. PEDF mRNA was localized in adult retina by in situ hybridization.
Results: In developing retinas (7.4 to 21.5 fetal weeks, Fwks), pAb anti-PEDF labeled retinal pigment epithelium (RPE) granules, developing cones, some neuroblasts and many cells in the ganglion cell layer (GCL). In adult retinas, pAb anti-PEDF labeled rod and cone cytoplasm and nuclei of rods but not cones. Cells in the INL and GCL, choroid, corneal epithelium and endothelium, and ciliary body were also pAb PEDF-positive. Preadsorption of pAb anti-PEDF with the immunizing peptide blocked specific labeling in retina and other tissues, except for photoreceptor outer segments. In agreement with the immunolocalization with pAb anti-PEDF, in situ hybridization revealed PEDF mRNA in the RPE, photoreceptors, inner nuclear layer cells and ganglion cells in adult retina. In developing retinas 18 Fwks and older, and in adult retinas, mAb anti-PEDF labeled the interphotoreceptor matrix (IPM). Western blots of retina, cornea, and ciliary body/iris with pAb anti-PEDF produced several bands at about 46 kDa. With mAb anti-PEDF, retina produced one band at about 46 kDa; cornea and ciliary body/iris had several bands at about 46 kDa.
Conclusions: PEDF, originally reported as a product of RPE cells, is present in photoreceptors and inner retinal cell types in developing and adult human eyes. Photoreceptors and RPE may secrete PEDF into the IPM.

Pigment epithelium-derived factor (PEDF) was first identified as a 50 kDa secreted protein in conditioned medium from cultured fetal human retinal pigment epithelium (RPE) cells [1,2]. The first evidence that PEDF might be involved in neuronal differentiation in the retina came from the observation that addition of crude soluble interphotoreceptor matrix (IPM) extracts containing PEDF or purified PEDF to human Y-79 retinoblastoma cells induced a neuronal phenotype, evidenced morphologically by extension from the cells of long, neurite-like processes and biochemically by increased expression of neuron-specific proteins [1-4]. More recently, PEDF was shown to have neurotrophic activity in the retina in vivo, as intravitreal injections of PEDF delayed apoptotic photoreceptor loss in mouse models of inherited retinal degeneration [5]. PEDF is also a neurotrophic and neuroprotective factor in other systems, including cultured cerebellar granule cells [6-8], primary hippocampal neurons [9], spinal cord motor neurons [10,11] and amphibian photoreceptors cultured in the absence of RPE cells [12]. The binding of PEDF to retinoblastoma and cerebellar granule cells may be receptor-mediated [13]. Recently, PEDF was shown to be a potent inhibitor of angiogenesis in endothelial cell cultures and in rat cornea and retina in vivo [14]. In addition, systemic application of PEDF prevented inner retinal neovascularization in a mouse model of retinopathy of prematurity [15].

The gene for PEDF was localized to chromosome 17p13 [16,17], near the locus of the RP13 gene for autosomal dominant retinitis pigmentosa (RP) and Leber congenital amaurosis [18]. However, disease-causing PEDF mutations have not yet been identified. PEDF shares sequence and structural homology with the serine protease inhibitor (serpin) family [3,19]. However, PEDF does not inhibit proteases and does not require the serpin reactive loop for its neurotrophic activity [20]. Northern analysis of RNA from bovine RPE and retina revealed expression of PEDF only by the RPE [21]. In situ hybridization studies demonstrated PEDF mRNA in the RPE of a human fetal eye at 17 weeks gestation [22], and Western blot analyses identified PEDF in soluble washes of the IPM from several species [4,22]. The protein is also synthesized and secreted by human ciliary epithelium [23]. Finally, PEDF gene expression has been documented in a broad range of fetal and adult human tissues, including brain and various endocrine organs [24].

In order to gain insight into the role of PEDF as a neurotrophic and anti-angiogenic factor in the retina, we have used immunofluorescence to localize PEDF in developing and adult human ocular tissues.

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METHODS

Tissue preparation: Post mortem normal adult human eyes were obtained from the University of Washington Lions Eye Bank (Seattle, WA) and the Foundation Fighting Blindness (Owings Mill, MD). Normal fetal human eyes were obtained from the University of Washington Human Embryology Laboratory (Seattle, WA). Informed consent was obtained from all adult donors ante mortem. All research was conducted in accordance with the Declaration of Helsinki and was approved by the institutional human subjects review boards of the University of Pennsylvania and the University of Washington.

The following specimens were evaluated: seven retinas aged 7.4 to 21.5 fetal weeks (Fwks) and three adult retinas aged 53, 65 and 75 years (Table 1). The anterior segments were also studied from the fetal eyes and two additional normal adult globes (Table 1). All globes were fixed for several weeks to months in 4% paraformaldehyde, with or without 0.5% glutaraldehyde, in 0.1 M phosphate buffer (pH 7.3) and stored thereafter in 2% paraformaldehyde in the same buffer.

Table 1. Characteristics of human retinas used for immunocytochemistry

Case No.	Age/Gender	PMI (h)	Fixative	Diagnosis
98-95	7.4 Fwk/NA	0	P	Normal
98-94	8.4 Fwk/NA	0	P	Normal
98-71	14.3 Fwk/NA	0	P	Normal
00-014	16.4 Fwk/NA	0	P	Normal
00-027	18 Fwk/NA	0	P	Normal
00-026	19 Fwk/NA	0	P	Normal
00-040	21.5 Fwk/NA	0	P	Normal
99-11-23	53 years/M	0	P	Normal
FFB-525	65 years/M	6.0	P + G	Normal
FFB-626	74 years/M	4.5	None	Normal
0164-00	75 years/M	4.25	P + G	Normal
FFB-641	78 years/F	20	None	Normal
0266-97	82 years/M	1.5	P	Normal
0823-95	NA	7.8	P	Normal

Characteristics of human retinas used for immunocytochemistry. PMI, post mortem interval; Fwk, fetal weeks; P, 4% paraformaldehyde; G, 0.5% glutaraldehyde; M, male; FFB, Foundation Fighting Blindness; NA, not available.

Immunocytochemistry: Retinal samples were cryosectioned at 12 µm and processed for immunofluorescence by published techniques [25]. Two antibodies against PEDF were used: an affinity-purified rabbit polyclonal antibody (pAb) to a synthetic peptide corresponding to amino acids 327-343 of the PEDF protein (1:200; Research Genetics, Inc., Huntsville, AL) and a mouse monoclonal antibody (mAb) to a recombinant GST fusion protein containing a 155 amino acid sequence at the C-terminal of human PEDF (1:2000; Chemicon International, Inc., Temecula, CA). The company, Chemicon, indicates that the mAb does not recognize GST. The epitope on PEDF recognized by this mAb is not shared with GST. Immunocytochemistry labeling with both the pAb and mAb were repeated over 10 times, always with the same results.

Double labeling was performed with antibodies to cell-specific proteins in rods, cones, the IPM, and neurons in the inner retina (Table 2). The secondary antibodies (goat anti-rabbit or anti-mouse IgG) were labeled with Alexa Fluor 488 (green; Molecular Probes, Eugene, OR), Cy-2 (green), or Cy-3 (red; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Nuclei were stained with propidium iodide (red, 2 µg/ml) or 4',6'-diamidino-2-phenylindole (blue, DAPI, 1 µg/ml), both from Molecular Probes. Control sections were treated in the same way with omission of primary antibody or preincubation of the primary antibody overnight at 4 °C with PEDF protein at a concentration of 50 µg/ml.

The immunolabeled sections were examined with a Leica DMR epifluorescence microscope (Deerfield, IL) and photographed with Kodak (Rochester, NY) Elite Chrome film, ASA 400, or with a laser scanning confocal microscope (BioRad MRC-600, Richmond, CA). Images were digitized with a flat bed scanner (Saphir HiRes, Heidelberg CPS GmbH, Bad Homburg, Germany) using LinoColor Elite 5.1 software (Heidelberg CPS GmbH), imported into a graphics program (Photoshop 5.0, Adobe, San Jose, CA) and dye-sublimation prints were generated.

Western blot analysis: Two normal, unfixed globes from donors aged 74 and 78 were frozen at 4.5 and 20 h post mortem,

Table 2. Cell-specific markers

Cell	Marker	Specificity	Species	Dilution	Source
Cones	7G6	50 kDa protein	mouse	1:250	P. MacLeish
Rods	4D2	rhodopsin	mouse	1:40	R. Molday
Rods	-	rhodopsin	rabbit	1:3,000	E. Kean
Interphotoreceptor matrix	-	interphotoreceptor retinoid-binding protein	rabbit	1:100	J. Saari
Interphotoreceptor matrix	-	interphotoreceptor retinoid-binding protein	mouse	1:2,000	J. Saari
Cones, horizontal, and amacrine cells	C8666	calbindin	mouse	1:200	Sigma
Horizontal, amacrine, and ganglion cells	P-3088	parvalbumin	mouse	1:10,000	Sigma

The above cell-specific antibodies were used immunocytochemistry in study.

respectively (Table 1). The globes were stored at -80 °C, thawed, and dissected immediately. The corneas, iris/ciliary bodies and retinas were processed for Western blot analysis using pAb or mAb anti-PEDF. Briefly, the tissue was homogenized in 0.09 M Tris-HCl (pH 7.4) containing 2.8% sodium dodecyl sulfate (SDS); 15 µg of pepstatin A, leupeptin, and aprotinin; 0.1 mM phenylmethylsulfonyl fluoride; and 2 mM EDTA. Protein concentration was determined using a BCA

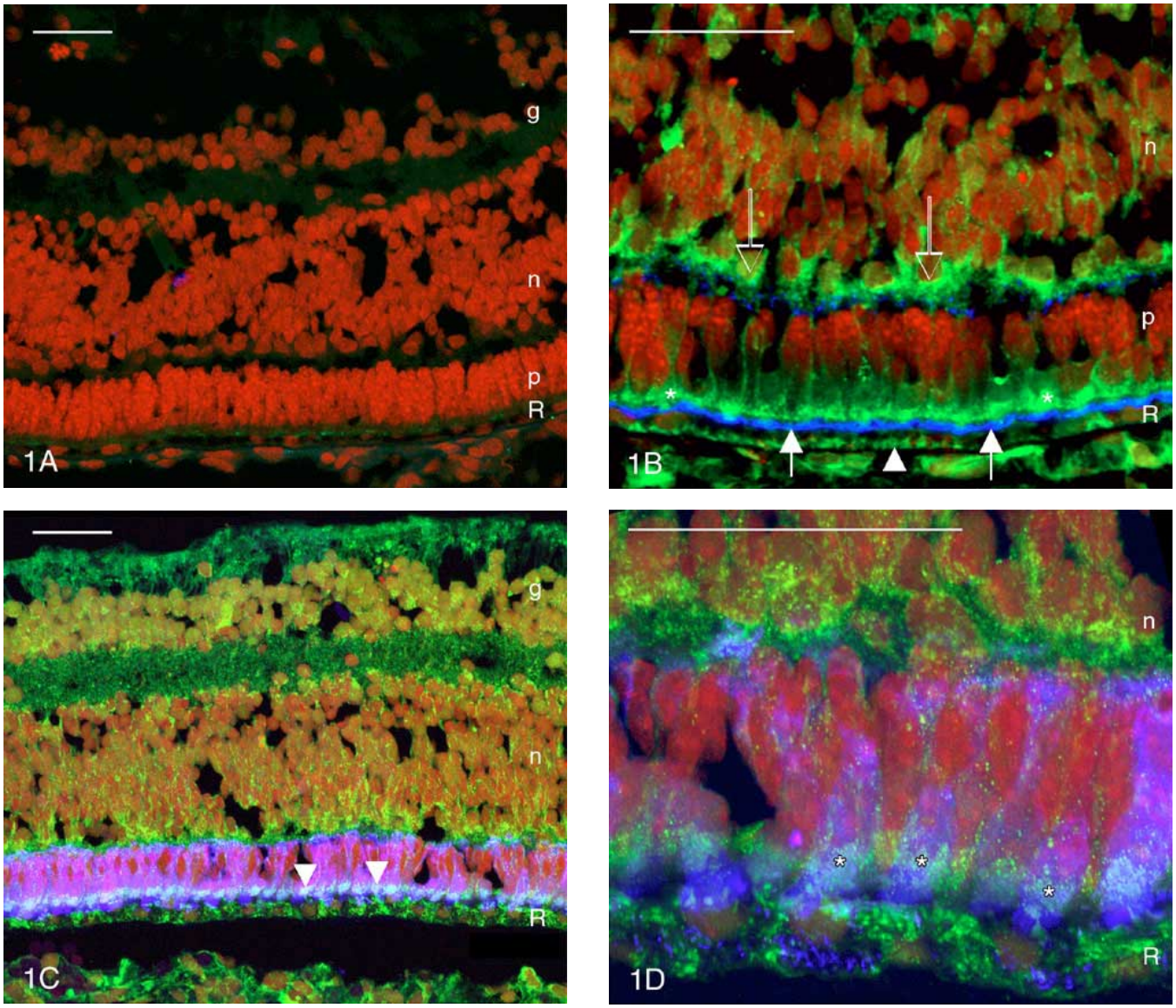

Figure 1. Immunocytochemical labeling of central part of developing human retina at age 21.5 Fwks. **A**: Control section treated with no primary antibody but with Cy-2- and Cy-5-labeled secondary antibodies shows lack of autofluorescence of the retina and RPE (R). p, differentiating photoreceptors; n, inner nuclear layer; g, ganglion cell layer. **B**: Double labeling with pAb anti-PEDF (green) and mAb anti-IRBP (blue). IRBP is restricted to a narrow band (arrow) of interphotoreceptor matrix between the photoreceptors (p) and retinal pigment epithelium (R). Note PEDF-positive inner segments (*) of the differentiating photoreceptors. PEDF labeling is also found in cytoplasmic granules in the RPE (arrowhead) and in cells (open arrows) in the outer part of the inner nuclear layer (n). **C**: Retina treated with the cone-specific mAb 7G6 (blue) and pAb anti-PEDF (green). Note cone inner segments (arrowheads) double labeled (cyan) with 7G6 and anti-PEDF. Many ganglion cells (g) are also PEDF-positive, as are scattered cells in the inner nuclear layer (n), especially the outer part. **D**: Higher magnification of **C**. Note cone inner segments (*) double labeled (cyan) with pAb anti-PEDF and mAb 7G6. PEDF-positive cells are present in the outer part of the inner nuclear layer (n). **E**: Developing rods are positive for rhodopsin (blue) but negative with pAb anti-PEDF (green). Note PEDF-positive cells in the ganglion cell (g) layer. **F**: Higher magnification of rods (arrowheads) that are positive with mAb anti-rhodopsin (blue) but negative with pAb anti-PEDF (green). **G**: Immunolabeling with pAb anti-PEDF (green) of fine granules (arrowheads) in the RPE (bracket). PEDF-labeling is also found in choroidal cells (c), but not in the developing horizontal cells, which have been labeled (blue) with anti-calbindin. **H**: Monoclonal anti-PEDF (blue) labels the interphotoreceptor matrix, which is also positive with pAb anti-IRBP (green) producing a cyan color. The cytoplasm of one cone (arrowhead) is positive with both anti-IRBP and anti-PEDF. Cell nuclei are stained (red) with propidium iodide. R, retinal pigment epithelium; p, photoreceptor layer; n, inner nuclear layer; g, ganglion cell layer. Bars = 40 µm.

reagent kit (Pierce, Rockford, IL). Eight µl of 0.09 M Tris-HCl and 40 µl of a second buffer (0.4% bromophenol blue and 4.7% 2-mercaptoethanol in 9.4% glycerol) were added to 92 µl of each sample containing 15 µg cornea, 30 µg iris and ciliary body, or 30 µg retina homogenate [26]. Samples were boiled (5 min) and each sample (140 µl) was electrophoresed on a 10% SDS polyacrylamide gel for 4 h at 60 mA.

Positive control protein was isolated from conditioned media of 293 cells, 48 h post-transfection with pCMV-PEDF-6xHis using a Xpress purification kit (Invitrogen, Carlsbad, CA). Briefly, ten 150 mm plates of 293 cells were transiently transfected with pCMV-PEDF-6xHis using the CalPhos mammalian Transfection kit (Clonetech, Palo Alto, CA). Forty-eight h post-transfection, the media were collected and incubated with 2 ml ProBond Resin (Invitrogen, Carlsbad, CA) for 3 h with agitation. PEDF-6xHis was eluted from the resin using increasing concentrations of imidazole (50 mM, 200 mM, 350 mM, and 500 mM). The pCMV-PEDF-6xHis plasmid had been generated by cutting pCEP4-PEDF (kindly provided by N. Bouck) [14] with *Sal*I, resulting in a cassette containing cDNA for 6xHis tagged human PEDF driven by a CMV promoter. This cassette was ligated (blunt ends) to psub201 [27] that had been digested with *Sna*B1 and *Eco*R1, followed by gel purification of the plasmid backbone. Accuracy of the cloning was confirmed by restriction digest and sequencing. Separated proteins were transferred to nitrocellulose membranes using a BioRad semi-dry transfer apparatus (1 h at 20 V), and were blocked for 10 min using 5% skim milk in incubation buffer (Tris-buffered saline, 0.1% Tween-20). Membranes were incubated overnight at 4 °C with rabbit pAb anti-PEDF (1:500) diluted in Tris buffer with 0.5% milk. The membranes were washed extensively with Tris buffer containing 0.1% Tween-20 and incubated for 2 h at room temperature with peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) at a dilution of 1:5000 in Tris buffer containing 0.5% milk. The membranes were washed extensively with Tris buffer containing 0.1% Tween-20 and treated with ECL Western blotting detection reagents (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, UK). Following ECL detection, the mem-

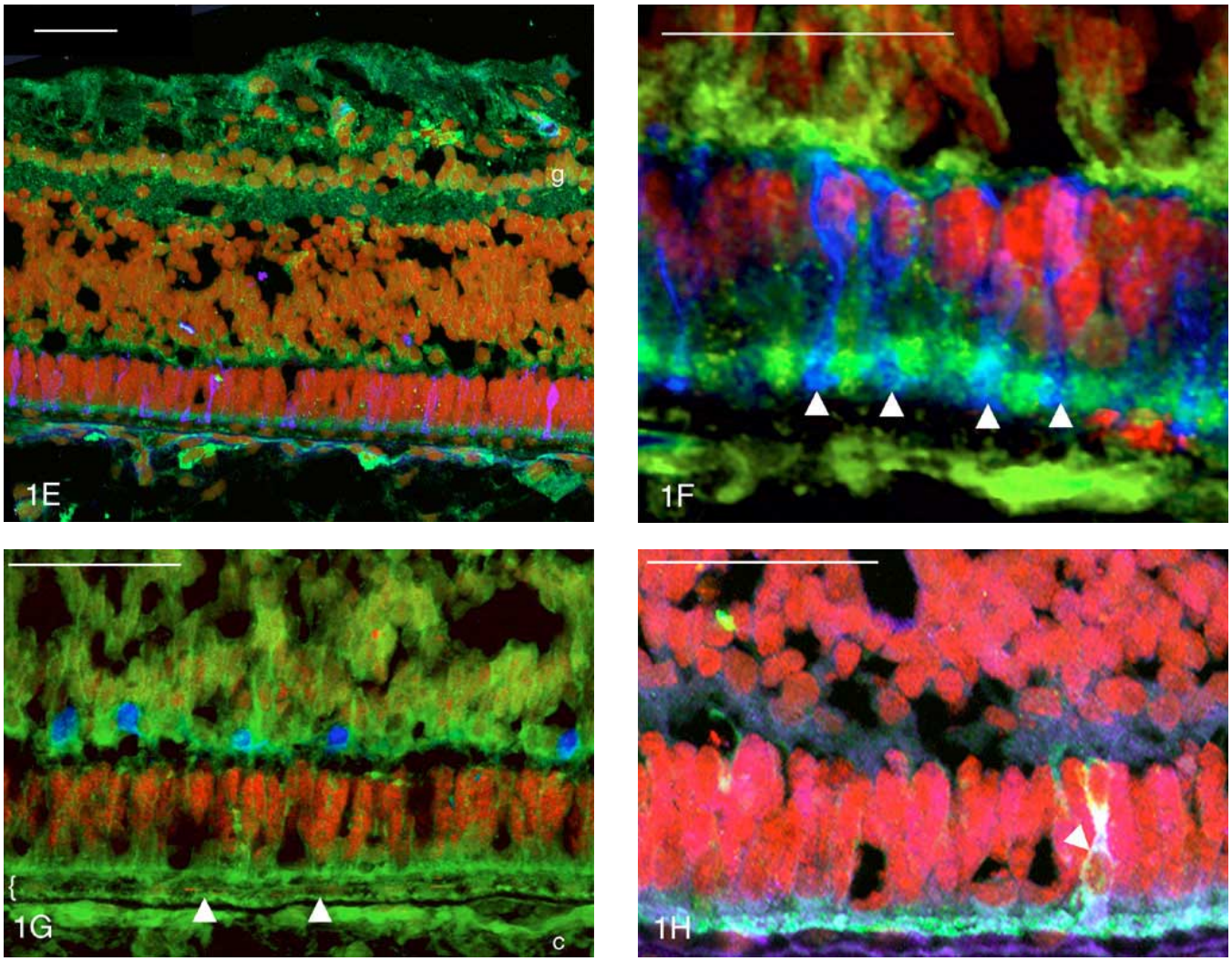

Figure 1, continued.

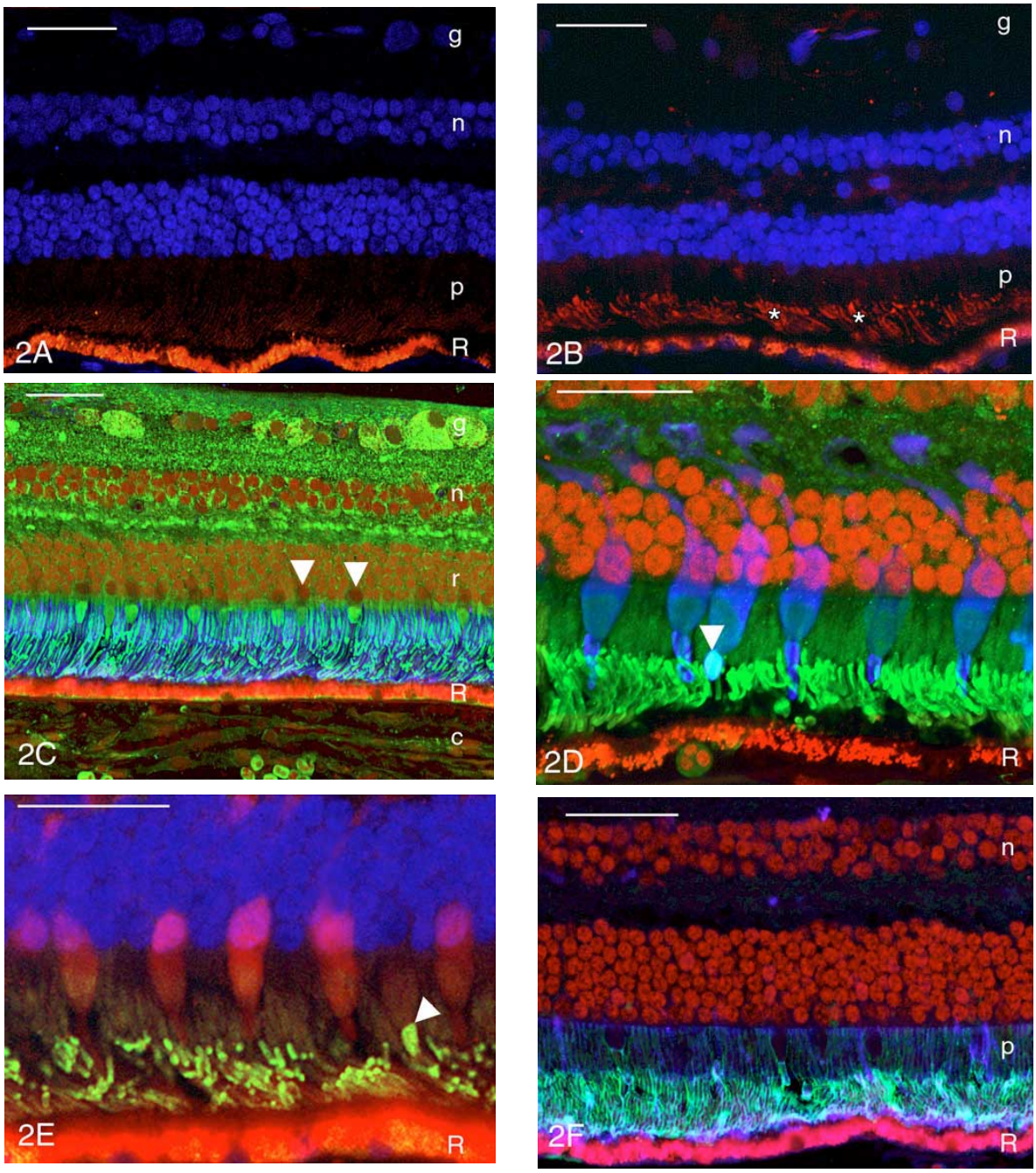

Figure 2. Immunocytochemical labeling of adult human retinas. **A**: Retina treated with no primary antibody but with Cy-2- (green) and Cy-5- (blue) labeled secondary antibodies shows strong autofluorescence of lipofuscin granules in the retinal pigment epithelium (R). p, photoreceptor layer; n, inner nuclear layer; g, ganglion cell layer. **B**: Retina treated with polyclonal anti-PEDF (green) that had been pre-adsorbed with the immunizing peptide. Note absence of label in photoreceptors (p), inner nuclear layer (n) and ganglion cell layer (g). However, some photoreceptor outer segments (*) are weakly positive, suggesting that this label may not be due to authentic PEDF. **C**: Polyclonal anti-PEDF (green) labels photoreceptor outer segments and rod and cone cytoplasm. The interphotoreceptor matrix is labeled (blue) with anti-IRBP. Note PEDF-positive rod nuclei (r) but PEDF-negative cone nuclei (arrowheads). The ganglion cells (g) and some cells in the inner nuclear layer (n) are labeled (green) with pAb anti-PEDF, as are cells in the choroid (c). **D**: Polyclonal anti-PEDF (green) labels the inner segments of the cones, which are labeled throughout with mAb 7G6 (blue), producing a cyan color. One cone outer segment (cyan, arrowhead) is positive with both pAb anti-PEDF and mAb 7G6. **E**: Polyclonal anti-PEDF (green) labels all rod outer segments and one cone outer segment (arrowhead). Monoclonal anti-calbindin (red) labels the cytoplasm of red and green sensitive cones, but not the cytoplasm of blue sensitive cones, identifying the cone with the PEDF-positive outer segment as a blue cone. **F**: Monoclonal anti-PEDF (blue) labels the interphotoreceptor matrix, which is also positive with pAb anti-IRBP (green), producing a cyan color. p, photoreceptor layer; n, inner nuclear layer. Cell nuclei are stained (red) with propidium iodide. R, retinal pigment epithelium; p, photoreceptor layer; n, inner nuclear layer; g, ganglion cell layer. Bars = 40 µm.

branes were stripped with 0.2 M glycine, pH 2.2 in 0.1% SDS in 1% Tween-20 and exposed to mAb anti-PEDF at 1:500 dilution. The remaining procedures were identical to those described above with the exception that a peroxidase-conjugated goat anti-mouse secondary antibody (1:5000) was used.

In situ hybridization: The pCMV-PEDF-6xHis (see above) was cut with *Nhe*I, blunted and then cut with EcoRI. The resultant fragment was gel purified and ligated to pBluescript KS(+/-) (Stratagene, La Jolla, CA) cut with *Bam*HI, blunted, and then cut with *Eco*RI. This plasmid was digested with *Kpn*I and religated in order to remove the 3' end of the cDNA, leaving a 356 bp insert. The plasmid was linearized with *Spe*I for antisense probe generation and *Nco*I for sense probe generation and transcribed with T3 and T7, respectively, using a DIG RNA labeling kit (Roche Pharmaceuticals, Indianapolis, IN). In situ hybridization was performed on 12 µm cryosections of normal human retina (99-11-23, a surgical specimen), as previously described [28], except the sections were digested in proteinase K for 20 min and the hybridization temperature was 65 °C.

RESULTS

Fetal human retinas: Fetal retina samples treated with secondary antibodies but no primary antibody showed little or no autofluorescence of the retina and RPE (Figure 1A). The antigen-adsorbed controls were also negative (not shown).

When possible, observations were made on the most central regions of the fetal retinas. Developing retinas aged 7.4 to 21.5 Fwks treated with pAb anti-PEDF showed strong labeling of a band in the outermost part of the retina (Figure 1B). Double labeling with pAb anti-PEDF and mAb anti-interphotoreceptor retinoid-binding protein (IRBP) demonstrated that the PEDF-positive band in the outermost retina was in the cytoplasm of the differentiating photoreceptors and not the IPM (Figure 1B). At ages 14.3 to 21.5 Fwks, the IRBP-positive band was thinner and external to the pAb PEDF-positive band (Figure 1B). Double labeling with the cone-specific marker, mAb 7G6, revealed that the PEDF-labeling was in the apical cytoplasm of the differentiating cones, including their developing inner segments at 21.5 Fwks (Figure 1C,D). Double labeling with pAb anti-PEDF and mAb anti-rhodopsin demonstrated small rods with rhodopsin-positive surface membranes at 21.5 Fwks; these rods were not labeled with pAb anti-PEDF (Figure 1E,F).

From 7.4 Fwks onward, pAb anti-PEDF also labeled fine granules in the RPE, scattered cells in the neuroblastic layer and numerous cells in the GCL (Figure 1B,C,D). From 18 Fwks onward, some pAb PEDF-labeled cells were present at the level of the horizontal cells in the outer part of the INL (Figure 1D). However, these PEDF-positive cells did not correspond to the differentiating horizontal cells that were labeled with anti-calbindin or anti-parvalbumin [29] (Figure 1G).

Developing retinas, ages 7.4 to 16.4 Fwks showed no labeling with mAb anti-PEDF. Treatment of fetal retinas at ages 18, 19 and 21.5 Fwks with mAb anti-PEDF produced a thin labeled band between the neural retina and RPE; this band was also positive with pAb anti-IRBP (Figure 1H). No labeling was seen with mAb anti-PEDF in the inner retina at these stages of development.

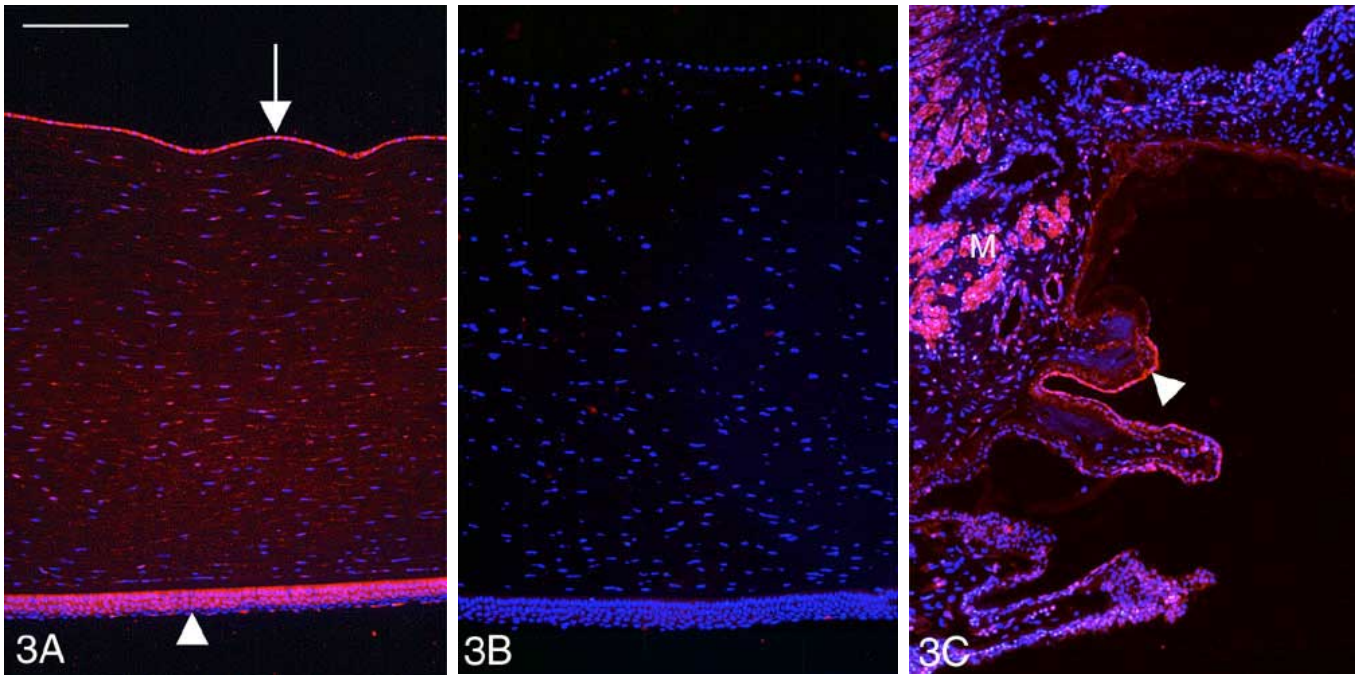

Figure 3. Immunocytochemical labeling of adult human anterior segment structures. **A**: Cornea treated with pAb anti-PEDF (red). Note intense labeling of the epithelium (arrowhead) and endothelium (arrow). **B**: Cornea treated with pre-adsorbed pAb anti-PEDF (red) shows no labeling. **C**: Ciliary body treated with pAb anti-PEDF (red). Note intense labeling of non-pigmented ciliary epithelium (arrowhead) and muscle (M). This immunolabeling was abolished by pre-adsorption of the pAb anti-PEDF. Cell nuclei are stained (blue) with DAPI. Bar = 100 µm.

Adult human retinas: Control sections treated with secondary antibodies but no primary antibody had strongly autofluorescent lipofuscin granules in the RPE (Figure 2A). Because of these highly autofluorescent granules, it was not possible to determine if other cytoplasmic areas of the adult RPE cells were reactive with mAb or pAb anti-PEDF. The antigen-adsorbed pAb anti-PEDF controls were negative except weak labeling of photoreceptor outer segments (OS, Figure 2B).

In the adult human retinas, all cells showed the expected labeling patterns with the cell-specific antibodies used in this study (see [30]). All cones had cytoplasmic and OS labeling with mAb 7G6, and all rod OS were labeled with both mAb (4D2) and pAb anti-rhodopsin. The IPM was labeled with both mAb and pAb anti-IRBP.

The pAb anti-PEDF produced strong immunolabeling of the rod OS and a few cone OS (Figure 2C,D). Most cone OS, identified by mAb 7G6, were PEDF-negative (Figure 2D). Double labeling with anti-PEDF and anti-calbindin, which labels the cytoplasm of red/green but not blue cones [31], revealed that the PEDF-positive cone OS were those of the blue cones (Figure 2E). However, weak labeling of the rod and blue cone OS persisted after the pAb anti-PEDF was adsorbed with the immunizing antigen (Figure 2B), indicating that this label may not be authentic PEDF.

The rod and cone inner segments and synapses were positive with pAb anti-PEDF, and the nuclei of the rods but not the cones were PEDF-positive (Figure 2C). Some cells in the INL and GCL had PEDF-positive cytoplasm but negative nuclei (Figure 2C). Discrete laminae in the inner plexiform layer and cells throughout the choroid were also positive for PEDF (Figure 2C). The labeling of the rod nuclei, INL and GCL cells, inner plexiform layer and choroid were absent with adsorbed pAb anti-PEDF (Figure 2B).

Adult retinas fixed with glutaraldehyde showed no immunolabeling with mAb anti-PEDF. Treatment with mAb anti-PEDF of the retina fixed with only paraformaldehyde labeled the IPM, identified by double labeling with anti-IRBP (Figure 2F).

Human anterior segments: The corneal epithelium and endothelium, ciliary body nonpigmented epithelium and muscle were strongly positive with pAb anti-PEDF from age 8.4 Fwks onward (not shown). The adult corneal epithelium and endothelium (Figure 3A) and the ciliary body non-pigmented epithelium and muscle (Figure 3C) were strongly positive with pAb anti-PEDF but were negative with mAb anti-PEDF. Tissues of the anterior segments were negative when no primary antibodies were used or when the pAb anti-PEDF was pre-adsorbed with PEDF (Figure 3B). As noted above, labeling of the IPM with mAb anti-PEDF was fixation-sensitive and not detectable in glutaraldehyde-fixed tissues. The anterior segments used for immunocytochemistry had been fixed with only paraformaldehyde, but this may have masked reactivity for PEDF, which was clearly detected in these tissues by Western analysis.

Western blotting: The same results were obtained with tissues from the two adult human eyes used for western blotting. The pAb anti-PEDF (Figure 4, "poly") recognized a band

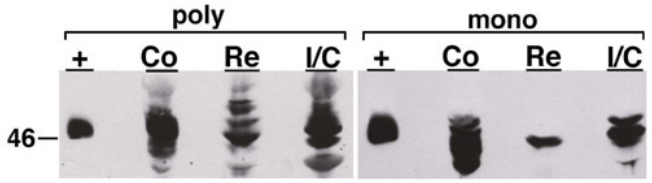

Figure 4. Western blot analysis of PEDF in human ocular tissues. Cornea (Co), neural retina (Re), and iris/ciliary body (I/C) were dissected from adult human eyes that had been frozen post mortem and stored at -80 °C. Protein from each tissue was loaded, along with the positive control protein ("+") obtained from transfected cells—see Methods. The blot was probed with pAb anti-PEDF ("poly"), stripped, and reprobed with mAb anti-PEDF ("mono"). Size (in kDa) is indicated to the left.

Figure 5. In situ hybridization with PEDF probes of sections of adult human retina. **A**: The antisense probe produces labeling of the RPE, photoreceptors, INL cells and GCL cells. **B**: The sense probe produces no specific labeling. The RPE contains yellow-gold lipofuscin granules and brown melanin granules. Bars = 40 µm.

at about 50 kDa corresponding to the positive control PEDF-6xHis fusion protein (Figure 4, "+"). The predominant band recognized by this antibody in cornea, retina and iris/ciliary body was about 46 kDa, although other bands close to this size were also present (Figure 4). The mAb anti-PEDF (Figure 4, "mono") produced similar results as the pAb except it recognized only one band at about 46 kDa in retina.

In situ hybridization: The antisense PEDF probe applied to sections of adult human retina produced labeling of the RPE, photoreceptors, INL cells and GCL cells (Figure 5A). Sections treated with the sense probe showed no specific labeling (Figure 5B).

DISCUSSION

To provide normal vision, the human eye contains precisely ordered neurons and blood vessels. Neurons of the retina are highly differentiated and organized into regular layers from late fetal development throughout adult life. The vasculature of the eye is also established early in development and maintenance of this blood supply is essential for normal visual function. Certain adult anterior segment structures (cornea, anterior chamber and lens) and the vitreous are normally free of blood vessels, as their transparency is essential for light to reach the retina. In the retina, branches of the central retinal blood vessels nourish the inner layers of the retina but do not penetrate into the vitreous. The photoreceptor layer is not vascularized, deriving metabolic support from the underlying RPE and choriocapillaris.

A number of angiogenic and anti-angiogenic factors have been identified in the eye and several of these appear to play major roles in normal development and in diseases. Basic fibroblast growth factor (bFGF), for example, is an angiogenic factor in many tissues and expression of this gene in the retina is known to be developmentally regulated [32] and altered by local injury [33]. Vascular endothelial growth factor (VEGF) is also found in the eye, and its levels parallel neovascularization disease states [34]. Experimental delivery of either of these two factors to ocular tissues produces dramatic neovascularization [35].

Similarly, several inhibitors of angiogenesis are normally present in the eye. A factor that has received a great deal of attention is PEDF, a protein originally identified in human RPE-conditioned medium [1,2]. PEDF inhibits endothelial cell migration and corneal neovascularization and has stronger anti-angiogenic activity than any of the other known angiogenesis inhibitors, including thrombospondins-1 and 2, endostatin, proliferin-related protein, retinoic acid and angiostatin. Notably, PEDF has 27 times more anti-angiogenic activity than angiostatin [14]. PEDF is also neurotrophic and promotes neuronal differentiation [1-3,16,19].

As a first step in evaluating the potential anti-angiogenic and neurotrophic properties of PEDF, we used immunocytochemistry to localize this protein at different stages of human ocular development. We found high levels of PEDF in developing and mature corneas, supporting data [14] that PEDF is the main anti-angiogenic factor in this tissue, and confirmed a previous report that PEDF is present in human ciliary body [23].

We also localized PEDF in developing human retinas. We had expected to find high levels of PEDF in the RPE, as this protein was first discovered in RPE-conditioned medium (see above), and mRNA for PEDF was identified in the RPE of a fetal human eye [22]. Our immunocytochemical results with pAb anti-PEDF suggest that the factor is synthesized by human RPE and cone cells, as well as cells of the inner retina, during development. Our findings with mAb anti-PEDF, which recognizes the C-terminal of PEDF, are consistent with secretion of PEDF into the IPM in fetal and adult human eyes.

In adult retinas, immunolabeling with pAb anti-PEDF demonstrated the protein in rods and cones, as well as cells of the inner retina (possible PEDF-reactivity in the RPE could not be determined because of the high autofluorescence of the lipofuscin granules). In agreement with the immunofluorescence labeling, mRNA for PEDF was localized to the RPE, photoreceptors, inner nuclear layer cells and ganglion cells in adult human retina by in situ hybridization (Figure 5) [36]. Immunolocalization of PEDF in the IPM of adult retinas by means of the mAb, which presumably recognizes a different epitope, agrees with previous biochemical studies and is consistent with the putative anti-angiogenic activity of PEDF. Changes in PEDF levels in this region in human maculas with AMD may contribute to development of choroidal neovascularization.

PEDF also promotes neuronal differentiation in vitro and survival in vivo. In photoreceptors, PEDF may function to maintain the highly differentiated state and survival of these fragile cells. Similar effects may occur in the inner layers of developing and adult human retinas. Many questions remained unanswered about the role(s) of PEDF in the eye [37]. Further studies, in progress, are aimed at evaluating changes in *PEDF* expression in retinal degenerative and neovascular disease states.

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