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Pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) in aged human choroid and eyes with age-related macular degeneration

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Abstract

The purpose of this study was to examine the localization and relative levels of vascular endothelial growth factor (VEGF; an angiogenic factor) and pigment epithelium-derived factor (PEDF; an antiangiogenic factor) in aged human choroid and to determine if the localization or their relative levels changed in age-related macular degeneration (AMD).

Ocular tissues were obtained from eight aged control donors (age range, 75–86 years; mean age, 79.8 years) with no evidence or history of chorioretinal disease and from 12 donors diagnosed with AMD (age range, 61–105 years; mean age, 83.9 years). Tissues were cryopreserved and streptavidin alkaline phosphatase immunohistochemistry was performed with rabbit polyclonal anti-human VEGF and rabbit polyclonal anti-human PEDF antibodies. Binding of the antibodies was blocked by preincubation of the antibody with an excess of recombinant human PEDF or VEGF peptide. Choroidal blood vessels were identified with mouse anti-human CD-34 antibody in adjacent tissue sections. Three independent observers graded the immunohistochemical reaction product.

The most prominent sites of VEGF and PEDF localization in aged control choroid were RPE–Bruch’s membrane–choriocapillaris complex including RPE basal lamina, intercapillary septa, and choroidal stroma. There was no significant difference in immunostaining intensity and localization of VEGF and PEDF in aged control choroids. The most intense VEGF immunoreactivity was observed in leukocytes within blood vessels. AMD choroid had a similar pattern and intensity of VEGF immunostaining to that observed in aged controls. However, PEDF immunoreactivity was significantly lower in RPE cells ($p = 0.0073$), RPE basal lamina ($p = 0.0141$), Bruch’s membrane ($p < 0.0001$), and choroidal stroma ($p = 0.0161$) of AMD choroids. The most intense PEDF immunoreactivity was observed in disciform scars. Drusen and basal laminar deposits (BLDs) were positive for VEGF and PEDF.

In aged control subjects, VEGF and PEDF immunostaining was the most intense in RPE–Bruch’s membrane–choriocapillaris complex. In AMD, PEDF was significantly lower in RPE cells, RPE basal lamina, Bruch’s membrane and choroidal stroma. These data suggest that a critical balance

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exists between PEDF and VEGF, and PEDF may counteract the angiogenic potential of VEGF. The decrease in PEDF may disrupt the balance and be permissive for the formation of choroidal neovascularization (CNV) in AMD.

Keywords

choroid; pigment epithelium-derived factor; vascular endothelial growth factor; RPE; Bruch's membrane; age-related macular degeneration; choroidal neovascularization

1. Introduction

Age-related macular degeneration (AMD) is one of the leading causes of irreversible visual loss among people 65 years of age and older in the western world (Ambati et al., 2003). The pathogenesis of AMD is probably multifactorial; its late onset, complex genetics, and strong environmental components may all contribute at some level. Choroidal neovascularization (CNV), growth of new blood vessels from the choroid, occurs in the advanced 'exudative' form of AMD and is the major risk factor for blindness. Exudative AMD is characterized by submacular ingrowth of choroidal vessels through a damaged Bruch's membrane that may remain beneath the retinal pigment epithelium (RPE) or breach the RPE layer and enter the subretinal space. The pathologic vasculature leaks serous fluid and/or blood and ultimately causes a disciform scar in and under the macular region of retina (Green and Key, 1977; Green et al., 1985). Unlike retinal neovascularization, CNV in AMD is not an obviously ischemia-driven disease. In fact, no unifying theory currently exists for the growth of abnormal blood vessels in the subretinal space in AMD and other related diseases. Ascertaining what initiates the growth of CNV in AMD is one of the main challenges in this field of research.

There is evidence to suggest that the net balance between endogenous angiogenic and anti-angiogenic growth factors exists in normal eye and an imbalance of these factors induces growth of new blood vessels inwards from the choroid. The presence of angiogenic growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) in surgically removed choroidal neovascular membranes and in experimental-induced CNV in animal models has been demonstrated (Amin et al., 1994; Kvant et al., 1996; Ishibashi et al., 1997). These previous studies also suggest that abnormalities of the extracellular matrix of RPE cells may promote a proangiogenic phenotype and the development of CNV.

In addition to proangiogenic factors, it has recently become apparent that a variety of endogenous antiangiogenic factors like pigment epithelium-derived factor (PEDF), endostatin, and thrombospondin (TSP) may contribute to vascular quiescence. PEDF, one of the endogenous antiangiogenic factors found in the eye, was first purified from the conditioned medium of human RPE cells (Tombran-Tink et al., 1991). It is a member of the serine protease inhibitor (serpin) family with neuroprotective, neurotrophic (Tombran-Tink et al., 1991), and antiangiogenic activities (Dawson et al., 1999). Its potential as a therapeutic agent for retinal and choroidal diseases triggered by photoreceptor degenerations and abnormal neovascularization has been explored in animal models (Mori et al., 2001,

2002a; Gehlbach et al., 2003) and is being evaluated in a clinical trial (Rasmussen et al., 2001). Although in vivo expression of PEDF in human choroid with AMD still remains to be elucidated, PEDF has been demonstrated in choroid of animal models and suggested to play an important role in experimental choroidal neovascular membrane formation (Ogata et al., 2002). The counterbalance of VEGF and PEDF is supported by the previous demonstrations that either inhibition of the VEGF system or over expression of PEDF inhibits choroidal neovascularization (Krzystolik et al., 2002; Mori et al., 2002b).

The goal of the current study was to determine the localization and relative levels of VEGF and PEDF in aged human choroid and RPE and to evaluate the changes in PEDF and VEGF localization and intensity in AMD. The data suggest that the levels of these two important endogenous proteins may regulate CNV.

2. Materials and methods

2.1. Donor eyes

Human donor eyes were obtained with the help of Janet Sunness, M.D. and Carol Applegate at the Wilmer Ophthalmological Institute (Baltimore, MD) and the National Disease Research Interchange (NDRI; Philadelphia, PA). Eyes of the following donors were used in the study: 12 subjects with AMD (age range, 61–105 years; mean age, 83.9 ± 13.1 years); eight aged control donors (age range, 75–86 years; mean age, 79.8 ± 3.7 years) with no medical history of chorioretinal disease. All donors were Caucasian. Table 1 includes the postmortem time (PMT) and death-to-enucleation time (DET), the age, sex, cause of death, and the medical and ocular history for each subject. The protocol of the study adhered to the tenets of the Declaration for Helsinki regarding research involving human tissue. The diagnosis of AMD was made by reviewing ocular medical history on the eye bank transmittal sheet and the postmortem gross examination of the eyecup using transmitted and reflected illumination with a dissecting microscope (Stemi, 2000; Carl Zeiss, Inc., Thornwood, NY).

2.2. Tissue preparation and sectioning

After a circumferential incision was made 0.5 cm posterior to the limbus, the anterior segment of the eye was removed, and the eyecup was examined by stereomicroscopy (Stemi 2000; Carl Zeiss, Inc., Thornwood, NY). Gross images were obtained with a digital camera (QImaging; Vancouver, BC, Canada) and imported directly into image analysis software (Photoshop ver. 6.0; Adobe Systems Inc., San Jose, CA, on a PowerMac G3; Apple Computer, Cupertino, CA). Eyes were fixed in 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at room temperature for 1 hr, cryopreserved with increasing concentrations of sucrose, and 8 μ m sections were cut from the macula as previously described (Lutty et al., 1993).

2.3. Immunohistochemistry

Streptavidin alkaline phosphatase (APase) immunohistochemistry was performed on cryopreserved tissue sections using a nitroblue tetrazolium (NBT) development system as previously described (Bhutto et al., 2004). In brief, 8 μ m thick cryosections were

permeabilized with absolute methanol and blocked with 2% normal goat serum in Tris-buffered saline (TBS; pH 7.4 with 1% BSA). Sections were also blocked with an avidin-biotin complex (ABC) blocking kit (Vector Laboratories, Inc., Burlingame, CA). After washing in TBS, the sections were incubated overnight at 4°C with a rabbit anti-human recombinant PEDF (1:60 000; P. Tong), a mouse anti-human PEDF (1:16 000; Chemicon, Temecula, CA), or a rabbit anti-human VEGF (1:8000; SC152, Santa Cruz, California) after dilution in TBS with 1% bovine serum albumen (BSA). Choroidal blood vessels were immunolabeled with mouse anti-human CD-34 (1:800; Signet Laboratory, Dedham, MA) antibody in adjacent tissue sections. After washing in TBS, tissue sections were incubated for 30 min at room temperature with appropriate biotinylated second-step antibodies diluted 1:500 (Kierkegaard and Perry, Gaithersburg, MD). Finally, sections were incubated with streptavidin alkaline phosphatase (1:500; Kierkegaard and Perry, Gaithersburg, MD) and then alkaline phosphatase activity was developed with a BCIP-NBT kit (Vector Laboratories, Inc., Burlingame, CA) with the addition of 1 mM (–)-Tetramisole HCl (Sigma), yielding a blue reaction product at sites of antibody binding. All immunohistochemical reagents, including antibodies, were identical for all specimens. At least three AMD and three aged normal subjects were processed simultaneously in each experiment.

To determine the specificity of VEGF and PEDF immunolabeling, VEGF antibody was preincubated overnight at 4°C with 100-fold molar excess of VEGF peptide used as an antigen and PEDF antibody was preincubated overnight with 100-fold molar excess of human recombinant PEDF before diluting the antibodies to their final concentration. The immunoreactivity from the preincubated antibodies was compared with the immunoreactivity from antibodies incubated overnight in BSA at the same concentration and with sections incubated with freshly diluted antibodies. Immunoreactivity from the polyclonal PEDF was also compared with immunoreactivity from the monoclonal anti-human PEDF peptide (Chemicon, Temecula, CA). All results presented in this study were generated using the polyclonal antibody.

2.4. PAS-APase staining method

A periodic acid Schiff's (PAS)/alkaline phosphatase (APase) staining method was used to identify viable choroidal capillaries (APase activity: blue reaction product) and basement membranes and basal laminar deposits (BLDs) (PAS staining: pink) as previously described (McLeod and Luty, 1994). In brief, the sections were incubated for 15 min in APase buffer at 37°C followed by washing in several changes of distilled water. Sections were placed into freshly prepared 0.5% periodic acid for 5 min followed by a brief wash in distilled water. The sections were then treated in Schiff's reagent for 10 min and developed in several changes of tap water until the water appeared clear. All reagents were purchased from Sigma-Aldrich (St Louis, MO) unless stated otherwise.

2.5. Tissue bleaching

For qualitative and quantitative assessment of immunohistochemistry at the level of choroid-Bruch's membrane-RPE complex, melanin from RPE and choroidal melanocytes was bleached by our published technique (Bhutto et al., 2004). In brief, sections were fixed in 4% paraformaldehyde overnight at 4°C immediately after streptavidin APase

immunohistochemistry. Slides were washed in distilled water, immersed in 0.05% potassium permanganate solution (Aldrich Chemical Co., Milwaukee, WI) for 25 min, and then rinsed in distilled water. Sections were covered with 35% peracetic acid (FMC Corp., Philadelphia, PA) in a humidified container for 20 min at room temperature followed by washing in distilled water. Finally cover slips were mounted with Kaiser's glycerogel.

2.6. Immunoreactivity grading system

Three independent masked observers, using a previously described grading system (Page et al., 1992; McLeod et al., 1995), graded the intensity of the immunoreactivity for each antibody in different structures. The intensity of labeling was graded qualitatively as: 8, uniformly intense immunoreactivity; 7, patchy and intense; 6, uniform and moderate; 5, patchy and moderate; 4, uniform and weak; 3, patchy and weak; 2, uniform and very weak; 1, patchy and very weak; and 0, none.

2.7. Statistical analysis

A mean score $\pm$ SD for each group was determined from the scores of all graders for each choroidal structure. The Gaussian distribution of these scores was demonstrated by the Kolmogorov and Smirnov method. The p values were determined by comparing mean scores from the aged control choroids with scores from eyes with AMD using the Students t -test and assuming unequal variance and two tails. A p value <0.05 was considered significant. All values represent mean $\pm$ SD. All statistical analysis was done with InStat software (version 2.0, GraphPad Software, San Diego, CA). 3.

Results

3.1. Immunolocalization of PEDF and VEGF in aged control choroid

Both PEDF and VEGF immunoreactivities were present in RPE-Bruch's membrane-choriocapillaris complex including RPE basal lamina, intercapillary septa, and choroidal stroma in aged control choroids. Immunostaining for VEGF was most prominent in choriocapillaris and intercapillary septa (Fig. 1(C)), whereas, PEDF staining was prominent around the intercapillary septa, and distinct but diffuse throughout choroidal stroma (Fig. 1(D)). The basal portion of RPE cells was positive for both VEGF and PEDF, whereas the apical surface of RPE cells had weak VEGF immunostaining. In large choroidal vessels, the immunostaining for both VEGF and PEDF was patchy and weak. Immunoreactivity for VEGF and PEDF was not uniform but rather heterogenous, so the graders scored a particular structure throughout the whole tissue section. In some choroidal tissue sections, presumed leukocytes within choroidal vascular lumens as well as in choroidal stroma were also intensely immunostained for VEGF.

The binding specificity of the VEGF and polyclonal antihuman PEDF antibodies was demonstrated by preincubating the antibodies with VEGF peptide or human recombinant PEDF before using it on sections. The preincubation eliminated most of the staining in the tissue (Fig. 2). The rabbit anti-human PEDF antibody demonstrated similar localization and staining intensity to a commercially available monoclonal antibody (data not shown) in choroid. All results shown and grades for immunoreactivity are from sections incubated with

polyclonal anti-human PEDF antibody. Using the same polyclonal anti-human PEDF antibody, we have reported PEDF-immunolabeling in sickle cell retina and choroid as well (Kim et al., 2003).

3.2. Immunolocalization of PEDF and VEGF in AMD choroid

Choroids of AMD eyes had a pattern and intensity of VEGF immunostaining similar to aged control eyes; RPE-Bruch's membrane-choriocapillaris complex including RPE basal lamina, intercapillary septa, and choroidal stroma had prominent VEGF immunoreactivity (compare Fig. 1(C) and 3(C)). There was no significant difference in the scores for localization of VEGF in choroidal structures between aged control and AMD eyes (Fig. 5(B)). PEDF immunoreactivity appeared lower in the choroid-Bruch's membrane-RPE complex of AMD eyes (Fig. 3(D)) compared with aged control eyes. In some choroidal tissue sections, expression of PEDF was variable in pathological and non-pathological areas of choroids in AMD eyes. In non-pathological areas of the same subject (case 11), the immunoreactivity for PEDF appeared comparable to VEGF in aged control eyes (compare Figs. 3 and 4). However, the immunoreactivity score for PEDF was significantly lower in RPE cells ($p = 0.0073$), RPE basal lamina ($p = 0.0141$), Bruch's membrane ($p < 0.0001$) and choroidal stroma ($p = 0.0161$) in AMD choroids than in aged control eyes (Fig. 5(A)). There was no significant difference in immunoreactivity scores for PEDF in choriocapillaris, intercapillary septa, and large choroidal vessels in AMD eyes compared with aged control eyes. All mean immunoreactivity scores for the choroidal structures of the aged control versus AMD eyes are shown in Fig. 5.

The specimens from an AMD eye (case 15) with apparent pigment epithelial detachment (PED) demonstrated that the BLD, inner choroid, and presumed leukocytes in the PED were intensely positive for VEGF, whereas PEDF immunoreactivity was most intense within the fluid within the space between the BLD and Bruch's membrane (Fig. 6). Another AMD eye (case 12) with geographic atrophy showed intense VEGF immunoreactivity associated with migrating cells in outer retina (Fig. 7(C)), whereas less intense PEDF was associated with migrating cells (Fig. 7(D)). Comparing the non-bleached APase/PAS stained section (Fig. 7(B)) to the bleached sections with VEGF and PEDF immunoreactivity (Fig. 7(C-D)), it appears that some of the VEGF positive cells are either pigment-laden macrophages or migrating RPE cells. BLDs were positive for both VEGF and PEDF immunoreactivity (Figs. 3 and 6). The identity of BLD was confirmed with PAS staining on serial sections (Figs. 3(B), 6(B)). In advanced AMD cases (subjects 16, 17, 18 and 20), disciform scars and small CNV formations were present. Disciform scars were the most intensely stained structures with PEDF (Fig. 8(D)), while CNV had only low levels or no PEDF immunoreactivity. The VEGF staining was prominent in CNV (Fig. 8(C)) as well as in the choroidal vessels including the choriocapillaris, whereas less intense VEGF immunoreactivity associated with disciform scar and choroidal stroma.

4. Discussion

This study demonstrates significantly lower PEDF immunoreactivity, a potent inhibitor of angiogenesis, in choroids of AMD subjects compared to the choroids of aged control

subjects. VEGF localization and immunoreactivity scores, however, were comparable between the two groups. The significantly lower levels of PEDF may create a permissive environment for choroidal neovascularization. The number of AMD subjects was not sufficient to divide the AMD group into subgroups with early and late stages; all AMD patient data was analysed in aggregate.

A striking finding in this study was that VEGF levels in choroids and RPE were not significantly higher in AMD subjects compared to aged control subjects. These results put AMD in striking contrast with diseases such as proliferative diabetic retinopathy or ischemic central retinal vein occlusion where VEGF levels in the vitreous are dramatically elevated compared with normal controls (Aiello et al., 1994; D'Amore, 1994). Numerous studies have investigated a possible role of VEGF in the pathogenesis of AMD related CNV (Kvanta et al., 1996; Lopez et al., 1996). One difference between those studies and ours is that none of CNV formations in our AMD cohort were in growth stages; all were present within disciform scars, and were likely quiescent. The disciform scar, which represents an endstage, is usually vascularized, almost invariably from the choroidal circulation but sometimes with retinal contributions as well, and can have both subretinal and sub-RPE components (Green, 1999). As the end stage, however, the CNV in scars is usually regressing or at least stabilized and not expanding. It is interesting that disciform scars had the most prominent localization of PEDF, except in the area of the scar adjacent to CNV.

Recently, numerous investigators have successfully induced CNV in animal models by overexpressing VEGF (Cui et al., 2000; Spilsbury et al., 2000; Schwesinger et al., 2001). Kwak and associates (Kwak et al., 2000) have demonstrated that blockade of VEGF activity results in inhibition of blood vessel growth in a murine model of laser-induced CNV. While the above studies suggest that VEGF may play a role in the development of CNV, it has been unclear whether VEGF in the choroid plays a primary pathogenic role in the natural history of this disease in humans. Moreover, other studies of VEGF in human AMD focused on the CNV membranes, not on the choroid and RPE in those subjects (Amin et al., 1994; Kvanta et al., 1996; Lopez et al., 1996). Another possibility is that VEGF produced by leukocytes may initiate CNV, which is suggested in a recent review by Cousins (Cousins and Csaky, 2002). Our results, however, which did not demonstrate greater VEGF in AMD, did not take into consideration the number of VEGF-positive leukocytes in AMD, but leukocytes certainly had high immunoreactivities of VEGF in all choroids. The VEGF family includes VEGF-A, VEGF-B, VEGF-C, and VEGF-D and placental growth factor (PlGF) (Neufeld et al., 1999). The presence of these proteins has recently been reported in human CNV membranes and experimental CNV (Otani et al., 2002; Rakic et al., 2003). The antibody used in the current study only recognizes VEGF-A, so our study sheds no light on the presence or absence of the other forms of VEGF in the tissue reported herein. Recently, a new VEGF-A isoform, VEGF_{165b}, has been isolated, which is an endogenous splice variant expressed in normal cells and tissues and in human plasma. This isoform is down-regulated in prostate cancer, binds VEGF receptor 2 with the same affinity as VEGF₁₆₅, but does not activate it or stimulate downstream signaling pathway. In two different in vivo angiogenesis models, this isoform is not angiogenic but rather inhibits VEGF₁₆₅-mediated angiogenesis, suggesting that regulation of VEGF splicing may be a critical switch from an antiangiogenic to a pro-angiogenic phenotype (Woolard et al., 2004). A more definitive answer concerning

the role VEGF could play in human AMD may come from ongoing prospective clinical trials that are evaluating inhibition of CNV due to AMD by injecting into the vitreous cavity an inhibitory antibody against VEGF (Genentech, 1999) or an aptamer that prevents VEGF binding its receptors (Vinores, 2003).

A previous report evaluated differentiated RPE cell expression levels of both VEGF and PEDF and the critical balance between VEGF and PEDF (Ohno-Matsui et al., 2001). The study provided evidence that VEGF upregulated the production of PEDF by human RPE cells via VEGFR-1 in an autocrine manner. This regulation leads to the restoration of a normal balance between angiogenic stimulators and inhibitors and this balance might have a key role in maintaining the homeostasis of the retinal vasculature. This interaction between VEGF and PEDF might be a specific feature of the RPE. RPE were the first cells known to produce PEDF. Several in vivo studies indicate that VEGF is produced constitutively by RPE cells in the normal eye (Adamis et al., 1993; Yi et al., 1998). Blaauwgeers et al. (1999) demonstrated that cultured human RPE cells preferentially secrete VEGF toward their basal (choroidal) surface, and that the VEGF receptor (VEGFR-2) is specifically localized at the retinal or apical surface of the choriocapillaris endothelium facing the RPE. Also, degeneration of RPE causes subsequent loss of fenestration in choroidal vascular endothelium (Neuhardt et al., 1999). These findings suggest that there is a paracrine relation between the choriocapillaris and RPE in the regulation of VEGF level, and VEGF might serve as a trophic factor for vascular endothelial cells or to maintain the fenestrated and highly permeable structure of choroidal vascular endothelium. This supports the current finding of prominent VEGF in normal choroids.

PEDF was recently identified as a major angiogenic inhibitor in the eye and its anti-angiogenesis potency is stronger than any other anti-angiogenesis factors (Dawson et al., 1999). Decreased PEDF levels in vitreous are associated with CNV in AMD patients (Holekamp et al., 2002). PEDF significantly suppresses VEGF-induced proliferation and migration of vascular endothelial cells (Duh et al., 2002). In this study, a significantly lower level of PEDF in choroid suggests that decreased levels of PEDF in the choroid-Bruch's membrane-RPE complex may be creating a more permissive environment for the formation of CNV in AMD. We have recently observed high endostatin immunoreactivity in Bruch's membrane of young human eyes (unpublished data) that declines with age and is further reduced in AMD choroids, compared with aged controls, while collagen XVIII levels were comparable in the two groups (Bhutto et al., 2004). Our work on endostatin and this study, suggests that endogenous anti-angiogenic agents, PEDF and endostatin, in Bruch's membrane may function to prevent the growth of CNV. This could explain why it is difficult to induce CNV in mouse except by laser injury, which damages and breaks Bruch's membrane. When VEGF is overexpressed from the rhodopsin promoter (Okamoto et al., 1997) or the RPE65 promoter (Schwesinger et al., 2001), the photoreceptor-produced VEGF induces neovascularization (NV) in outer retina and RPE-produced VEGF induces intrachoroidal NV. Neither NV penetrated and crossed Bruch's membrane, which contains high levels of endostatin and PEDF. Therefore, our findings indicate that two inhibitors of angiogenesis, PEDF and endostatin, prominently present in Bruch's membrane and around choriocapillaris, are reduced in AMD. Based on immunoreactivity, the normal level of

VEGF present in the area may be sufficient to stimulate new vessel growth when two natural antagonists are reduced or absent.

We speculate that in normal healthy conditions, the RPE has a positive survival effect on the maintenance of the highly vascularized, highly permeable fenestrated choriocapillaris on its outer basal aspect by secreting VEGF, while maintaining the photoreceptor layer internal to the RPE completely avascular and preventing the development of CNV in the subretinal space through VEGF-induced PEDF upregulation in an autocrine manner.

In conclusion, VEGF and PEDF immunostaining in aged control choroids was most intense in RPE-Bruch's membrane-choriocapillaris complex. In AMD, PEDF was lower in RPE cells and Bruch's membrane. These data suggest that a critical balance exists between endogenous antiangiogenic agents like PEDF and endostatin (Bhutto et al., 2004), and VEGF, and the antiangiogenic factors may counteract the angiogenic potential of VEGF. Under pathological conditions, PEDF and endostatin decrease to disrupt this balance. This equilibrium shift may be significant in permitting pathological processes involving RPE cells, RPE basal lamina and Bruch's membrane to occur and also permit choroidal neovascularization to form in AMD.

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Fig. 1.

Sections of submacular choroid incubated with VEGF and PEDF antibodies from an aged control eye (case 8). (A) Immunostaining of CD-34 was associated with choroidal vessels including the choriocapillaris. (B) Blue APase reaction product was present in the choriocapillaris and large choroidal vessels and pink PAS staining in Bruch's membrane and vascular basement membrane. Immunostaining of VEGF (C) and PEDF (D) is prominent in RPE basal lamina in some areas, Bruch's membrane, choriocapillaris basement membrane, intercapillary septa, and less intensively positive in choroidal stroma. Pigment in the sections (A, C, D) was bleached from RPE and choroidal melanocytes.

Fig. 2. Choroid sections from an aged control eye (case 7) immunostained with rabbit anti-human VEGF (A) and rabbit anti-human PEDF (B) preincubated overnight with BSA showed prominent labeling of Bruch's membrane, choriocapillaris basement membrane, and intercapillary septa. The intensity of immunoreaction product for PEDF was observed higher than for VEGF. These were considered 'mock-blocked' samples, because they were preincubated with a control protein, BSA. The choroidal immunoreactivity was almost eliminated by preincubating the antibodies overnight with antigens, VEGF peptide (C) and the recombinant human PEDF (D). Intense VEGF and PEDF immunoreactivity was associated with drusen (arrows). RPE (arrowheads) also had PEDF immunoreactivity. (Asterisk, choroidal vessels).

Fig. 3. Sections of submacular choroid from an AMD eye (case 11). (A) Immunostaining of CD-34 shows well-arranged choriocapillaris, while PAS staining (B) shows BLDs (arrows). Immunostaining of VEGF (C) shows intense immunoreactivity in choroidal structures, whereas PEDF immunoreactivity (D) is less intense compared to VEGF. BLDs (arrows) are also positive for VEGF, whereas, the PEDF is only weakly associated with BLDs.

Fig. 4. Immunolocalization of VEGF and PEDF in AMD eye (case 11), the same subject as in Fig. 3. Sections are from choroid nasal to the optic nerve head, a non-pathological area. (A) Immunostaining of CD-34 associated with choriocapillaris, whereas APase/PAS staining (B) shows normal choroidal morphology. Immunostaining of VEGF (C) and PEDF (D) antibodies appeared identical to aged control eyes.

Fig. 5.

Mean immunoreactivity scores $\pm$ SD for choroidal structures of aged control (black) and AMD (white) eyes. The immunoreactivity scores for PEDF (A) were significantly decreased in RPE ($p = 0.0073$), RPE basal lamina ($p = 0.0141$), Bruch's membrane ($p < 0.0001$), and choroidal stroma ($p = 0.0161$) in AMD eyes compared with the aged control eyes. There was no significant difference in the VEGF immunoreactivity scores (B) for choroidal structures between AMD eyes and aged control eyes ($p = 0.4444$).

Fig. 6. AMD eye (case 15) with pigment epithelial detachment (PED;*). Immunostaining of CD-34 (A) associated with choroidal vessels with prominent number of choriocapillaris dropout under the PED. PAS staining (B) shows BLD, serous fluid and leukocyte component (arrows in B, C, and D) in PED. Note the BLD, inner choroid, and leukocytes in the PED intensely positive for VEGF (C), whereas PEDF immunoreactivity (D) is most intense in the fluid within the space between the BLD and Bruch's membrane.

Fig. 7. Sections of retina and choroid from an AMD eye (case 12) show intense VEGF (C) immunoreactivity associated with migrating cells (arrowheads), whereas the PEDF (D) is less intense in cells, but more intense in BLDs. PAS (B) staining clearly demonstrates the cells (arrowheads) and the BLD. CD-34 (A) shows obvious choriocapillaris dropout.

Fig. 8. Sections of choroid from AMD eye (case 16) with a disciform scar with small CNV formation within the scar. CD-34 (A) and APase (B) labeling demonstrates the viable CNV (arrows) and pink PAS staining in scar (asterisk). The CNV has intense VEGF immunoreactivity (C) associated with it (arrows), whereas the PEDF (D) is negative in CNV. The scar (asterisk) has much more PEDF than VEGF. Also note that PEDF immunoreactivity in choroid is less intense compared to the VEGF.

Table 1

Characteristics of human donor cryopreserved eyes

Case #	Time (hr)	Age/race/ sex	Primary cause of death	Medical history	Ocular diagnosis	Ocular history
Aged	DET	PMT				
1	2.5	33	75/CF	Heart disease	Normal	None
2	7	27	76/CF	Lung CA	Normal	None
3	1	26	77/CM	COPD	Normal	Unknown
4	2.5	28	80/CM	COPD	Normal	Cataract-OU
5	7.15	28	80/CM	Intracranial Hemorrhage	Normal	IOL-OU
6	3	15	82/CM	Metastasis Brain CA	Normal	None
7	3	16	83/CM	Cardiac respiratory arrest	Normal	IOL-OU
8	5	26	86/CF	Respiratory failure	Normal	None
<i>AMD</i>						
9	3.5	34	61/CM	Metastasis esophageal CA	AMD, early	Radial keratotomy
10	3.5	42	69/CF	Subarachnoid Hemorrhage	AMD (GA), late	Macular degeneration
11	4	33	74/CM	Prostate CA	AMD, early	Macular degeneration; IOL-OU
12	7	30	75/CM	Aspiration pneumonia	AMD (GA), late [*]	Macular degeneration-OS
13	3	33	79/CM	Pneumonia	AMD, early	Macular degeneration; IOL-OS
14	5	29	81/CF	Myocardial Infarction	AMD, early	Macular hole-OD
15	3	12	83/CM	Prostate CA	AMD, early	Cataract, maculopathy-OU
16	4	20	93/CF	Multi system failure	AMD (Disc. Scar), late [*]	Macular degeneration-OU
17	3	36	94/CM	Cardiac failure	AMD (Disc. Scar), late [*]	Macular degeneration; IOL-OS
18	3.5	?	95/CM	Cardiomyopathy	AMD (Disc. Scar), late [*]	Legally blind-OU
19	2	33	98/CF	Old age	AMD, early	IOL-OD
20	4.5-5	11	105/CM	COPD	AMD (Disc. Scar),	Unknown

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Case #	Time (hr)		Age/race/ sex	Primary cause of death	Medical history	Ocular diagnosis	Ocular history
	Aged	PMT					
GA), late							
DET, Death to enucleation time; PMT, Postmortem time (death to fixation); C, Caucasian; M, Male; F, Female; AMD, Age-related macular degeneration; DM, diabetes mellitus; HTN, Hypertension; COPD, chronic obstructive pulmonary disease; CA, cancer; GA, Geographic atrophy; IOL, Intra-ocular lens;							
* choroidal neovascularization (CNV); OU, both eyes; OD, right eye; OS, left eye.							