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Reduction of Endogenous Angiogenesis Inhibitors in Bruch's Membrane of the Submacular Region in Eyes With Age-Related Macular Degeneration

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Abstract

Objectives—To determine the relative levels of 3 potent inhibitors of angiogenesis (endostatin, pigment epithelium–derived factor, and thrombospondin 1) in the retinal pigment epithelium–Bruch's membrane–choriocapillaris complex in the submacular region in aged control eyes and eyes with age-related macular degeneration (AMD).

Methods—Immunohistochemical analysis with antibodies against endostatin, pigment epithelium–derived factor, and thrombospondin 1 was performed on the macular region of aged control donor eyes (n=8; mean age, 79.8 years) and eyes with AMD (n=12; mean age, 83.9 years). Three independent masked observers scored the reaction product (scored from 0–7). Mean scores from the control eyes and the eyes with AMD were analyzed using 1-way analysis of variance and unpaired *t* test.

Results—In control eyes, strong immunoreactivity of all 3 inhibitors was observed in the retinal pigment epithelium–Bruch's membrane–choriocapillaris complex. Immunoreactivity for endostatin, pigment epithelium–derived factor, and thrombospondin 1 in Bruch's membrane was significantly lower in eyes with AMD compared with aged control eyes (analysis of variance, $P=.003$, $P=.009$, and $P<.001$, respectively). In the choriocapillaris, a significant reduction was observed in endostatin (analysis of variance, $P=.02$) and thrombospondin 1 (analysis of variance, $P=.005$) in eyes with AMD.

Conclusions—These findings suggest that endogenous angiogenesis inhibitors in the retinal pigment epithelium–Bruch's membrane–choriocapillaris complex may provide a biochemical barrier for choroidal neovascular invasion.

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Additional Information: The eFigure is available at http://www.archophthalmol.com.

Clinical Relevance—Decreased levels of angiogenic inhibitors at the retinal pigment epithelium–Bruch's membrane–choriocapillaris complex in eyes with AMD make Bruch's membrane vulnerable to choroidal neovascularization.

Choroidal Neovascularization (CNV) is a major cause of vision loss in patients with age-related macular degeneration (AMD).[1] Because the exact mechanism underlying the pathogenesis of CNV is still poorly understood, identifying risk factors and preventive strategies is important to decrease the effect and burden of blindness from this condition. Several theories of pathogenesis have been proposed, including retinal pigment epithelium (RPE) dysfunction, alterations in Bruch's membrane, oxidative stress, genetic defects, ocular perfusion abnormalities, inflammatory processes, and ischemia.[2,3]

Bruch's membrane is a stratified extracellular matrix complex that functions as a physical as well as biochemical barrier for normal physiologic processes and pathological processes like CNV. Disruption of or damage to this barrier often results in the growth of CNV into the sub-RPE and/or subretinal spaces. Age-related changes in Bruch's membrane have been studied to ascertain those factors that determine progression to AMD in some individuals. At present, the determinants of Bruch's membrane changes that predispose to CNV are unclear. New vessel formation is thought to occur as a consequence of an imbalance in the stimulating and inhibiting influences of growth factors, and any disruption to growth factor diffusion through Bruch's membrane to the choroid could alter this balance. Evidence has been found for a similar mechanism in laser-induced CNV, supporting the hypothesis that an imbalance between angiogenic stimulators and inhibitors is a cause of pathological neovascularization.[4]

Angiogenesis, as occurs in CNV, is tightly controlled by a dynamic balance between positive and negative regulators. In most quiescent healthy tissues, inhibitory influences predominate and vessels remain stable. In contrast, in a variety of pathological states such as neovascular AMD, neovascularization occurs because of decreased production of inhibitors and/or increased production of angiogenic stimulators.[5] There is considerable evidence that vascular endothelial growth factor A is a prime regulator of angiogenesis.[6] Endogenous negative regulators have been identified as well, including thrombospondin 1 (TSP-1), angiostatin and endostatin, and pigment epithelium–derived factor (PEDF).[7–9]

We have previously described the immunohistochemical localization of 3 endogenous angiogenesis inhibitors (endostatin, PEDF, and TSP-1) individually in submacular choroids of eyes with AMD.[10–12] These inhibitors are predominantly extracellular proteins that are part of the matrix or bind to the matrix. The purpose of this study was to collectively determine whether the relative levels of these 3 potent inhibitors were significantly changed in individual subjects with AMD as well as in eyes with AMD as a group. We have primarily focused on the sub-macular RPE–Bruch's membrane–choriocapillaris (CC) complex, which represents the extracellular matrix environment for the basal surface of RPE cells and functions as a physical as well as biochemical barrier for normal physiologic and pathological processes.

METHODS

DONOR EYES

Eight eyes from human aged donors (age range, 75–86 years; mean age, 79.8 years) with no evidence or clinical history of age-related changes in the macula and 12 eyes from donors with early and late AMD (age range, 61–105 years; mean age, 83.9 years) were used. Human donor eyes were obtained with the help of Janet Sunness, MD (Greater Baltimore Medical Center, Baltimore, Maryland), Carol Applegate (Wilmer Ophthalmological Institute, Baltimore), and the National Disease Research Interchange (Philadelphia, Pennsylvania). Donor eyes were shipped to the Wilmer Ophthalmological Institute in wet gauze sponges at 4°C. Table 1 shows the characteristics of each human donor subject used in this analysis. All of the donors were white. The diagnosis of AMD was made by reviewing ocular medical history (if available) and the postmortem gross examination results of the posterior eyecup. The protocol of the study adhered to the tenets of the Declaration of Helsinki regarding research involving human tissue and was approved by the Johns Hopkins Medicine Institutional Review Boards.

TISSUE PREPARATION

The posterior eyecups were fixed in 2% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.4) with 5% sucrose at room temperature for 1 hour. The tissue was cut into culottes of the vitreous–retina–choroid complex and cryopreserved as previously described.[13] Serial 8-μm cryosections were cut from the inferior macula, collected in duplicate on glass slides coated with Vectabond (Vector Laboratories, Inc, Burlingame, California), dried, and stored at −80°C.

ALKALINE PHOSPHATASE IMMUNOHISTOCHEMICAL ANALYSIS

Streptavidin alkaline phosphatase immunohistochemical analysis was performed on cryosections using a nitroblue tetrazolium development system as previously described.[10] The sections were incubated overnight at 4°C with one of the following primary antibodies: goat antihuman endostatin (dilution, 1:4000; R&D Systems, Minneapolis, Minnesota), rabbit antihuman recombinant PEDF (dilution, 1:60 000; graciously provided by Patrick Tong, MD, PhD, at Wilmer Ophthalmological Institute), and mouse antihuman TSP-1 (dilution, 1:100; Abcam, Cambridge, Massachusetts). Blood vessels were immunolabeled in adjacent sections with mouse antihuman CD34 (dilution, 1:800; Signet Laboratory, Dedham, Massachusetts). Alkaline phosphatase was developed with a 5-bromo-4-chloro-3-indoyl phosphate–nitroblue tetrazolium kit (Vector Laboratories, Inc), yielding a blue immunoreaction product. To demonstrate the validity of antibody binding, antiendostatin and anti-PEDF antibodies were preincubated with a 200-fold excess of peptide or human serum albumen overnight at 4°C before use.[10,11] As a negative control, the primary antibodies were omitted or a nonimmune IgG was used at the same protein concentration as the primary antibody.[12] Melanin pigment in RPE and choroidal melanocytes was bleached as described previously.[10] Hematoxylin and eosin staining was used to examine the morphology of the choroid in aged control eyes and eyes with AMD.

Three independent masked observers (I.A.B., K.U., and G.A.L.) scored the relative intensity of the immunoreactivity for each antibody in choroidal structures using a previously described 7-point grading system.[14,15]

STATISTICAL ANALYSIS

Statistical analysis was performed using InStat version 2.0 software (GraphPad Software, San Diego, California) and SAS version 9 commercial statistical software (SAS Institute, Inc, Cary, North Carolina). A mean (standard deviation) score for each group (aged control, early AMD, and late AMD) was determined from the scores of all of the graders for each choroidal structure. The *P* values were determined by comparing mean scores from the aged control eyes with scores from eyes with AMD using 1-way analysis of variance, and $P<.05$ was considered statistically significant. A post hoc *t* test with Bonferroni adjustment was used to compare mean scores from each group whenever applicable.

RESULTS

IMMUNOLOCALIZATION OF ANGIOGENESIS INHIBITORS IN THE RPE–BRUCH'S MEMBRANE–CC COMPLEX OF AGED CONTROL CHOROID

The immunostaining for TSP-1, endostatin, and PEDF was present in the RPE–Bruch's membrane–CC complex, including the RPE basal lamina, intercapillary septa, and choroidal stroma in each healthy aged control donor subject. Bruch's membrane had the most prominent TSP-1 (Figure 1B and C, subject 4), endostatin (Figure 2A and B), and PEDF (Figure 3B and C) immunoreactivity. At higher magnification, the basal lamina of RPE cells was intensely labeled for endostatin (Figure 2B) and PEDF (Figure 3C). Strong immunoreactivity for endostatin (Figure 2A and B) and PEDF (Figure 3B and C) was also observed in the CC. Large choroidal blood vessels, intercapillary septa, and the choroidal stroma had moderate labeling for endostatin and PEDF, but TSP-1 labeling was very weak in these structures. The TSP-1 immunoreactivity had a uniform distribution in each aged control donor tissue. However, immunostaining for endostatin and PEDF was not uniform but rather heterogeneous. Staining with CD34 was localized in endothelial cells of the CC and large choroidal vessels (Figure 3A). Hematoxylin and eosin staining showed normal morphological features of the choroid (Figure 1A and Figure 2C).

IMMUNOLOCALIZATION OF ANGIOGENESIS INHIBITORS IN THE CHOROID OF EYES WITH AMD

Eyes With Early AMD—Representative examples of several characteristics of eyes with early and late AMD are shown in Figure 1 and Figure 2. Drusen and basal laminar deposits were routinely observed in eyes with AMD (Figure 2F), and the CC lumens appeared constricted and irregular (Figure 3G). In the sections stained with hematoxylin and eosin, the eosin stained the basal laminar deposits intensely pink in eyes with early AMD (Figure 2F). In a representative eye with early AMD (subject 10), weak TSP-1 (Figure 1E and F) and endostatin (Figure 2D and E) expressions were observed in the RPE–Bruch's membrane–CC complex, whereas the pattern and intensity of PEDF immunostaining appeared comparable to the healthy aged control choroid (Figure 3E and F). The scores for TSP-1, endostatin, and PEDF were significantly lower in Bruch's membrane in eyes with early AMD compared

with aged control eyes (Figure 4). However, the endostatin immunoreactivity score in Bruch's membrane was not significantly different in eyes with early AMD compared with eyes with late AMD. In the RPE basal lamina, such reduction was observed in endostatin only (Figure 4). In the CC, immunostaining for TSP-1 and endostatin was significantly weaker in eyes with early AMD compared with aged control eyes. Basal laminar deposits were intensely immunoreactive with PEDF (Figure 3E and F). Four of 6 eyes with early AMD had lower scores for all of the 3 inhibitors in Bruch's membrane than the corresponding mean score of the aged control eyes. In 1-way analysis of variance, comparison of the RPE–Bruch's membrane–CC complex scores between aged controls, eyes with early AMD, and eyes with late AMD showed a statistically significant difference among the 3 groups in all of the parameters except the RPE basal lamina (Table 2).

Eyes With Late AMD—In the example of the choroid in an eye with late AMD (subject 19) (Figure 1), negative to very weak TSP-1 (Figure 1H and I), endostatin (Figure 2G and H), and PEDF (Figure 3H and I) staining was observed in Bruch's membrane. The immunoreaction for endostatin and PEDF appeared more diffuse in the choroidal stroma (Figure 2H and Figure 3I). Hematoxylin and eosin staining demonstrated the morphological changes in the retina and choroid, such as migration of RPE cells into the retina in late AMD (Figure 1G and Figure 2I). Localization of CD34 demonstrated a highly constricted CC in some areas with some lumens not positive for CD34, suggesting no viable endothelial cells (Figure 3G).

The eyes with late AMD had a significantly lower score for endostatin, PEDF, and TSP-1 in Bruch's membrane when compared with the healthy aged control eyes (Figure 4). In the CC, a significant reduction was observed in endostatin and TSP-1. In the RPE basal lamina, immunoreactivity for the 3 inhibitors appeared not quite significant in eyes with late AMD compared with aged control eyes. With increasing severity of AMD (late AMD vs early AMD), the scores for PEDF and TSP-1 in Bruch's membrane significantly declined in the eyes with late AMD compared with the eyes with early AMD. However, the differences in the RPE basal lamina and CC were not significant between the eyes with late and early AMD when the post hoc test (Bonferroni) was applied. Mean immunoreactivity scores for the RPE–Bruch's membrane–CC complex of eyes with AMD vs aged control eyes are shown in the eFigure (available at http://www.archophthalmol.com).

When we compared the mean scores of the 3 groups (aged control, early AMD, and late AMD) by analysis of variance, all of the 3 inhibitors (endostatin, PEDF, and TSP-1) in all of the 3 groups were statistically significantly different from each other in Bruch's membrane, whereas endostatin and TSP-1 were significantly lower in the CC (Table 2).

Considering the immunoreactivity scores for the 3 inhibitors in the RPE–Bruch's membrane–CC complex in the eyes with early and late AMD, 67% (4 of 6) of the eyes with early AMD and 100% (6 of 6) of the eyes with late AMD had lower scores for all of the 3 inhibitors in Bruch's membrane. Fifty percent (6 of 12) of eyes with early and late AMD had lower scores for all of the 3 inhibitors in the RPE basal lamina and the CC.

COMMENT

In this study, our immunostaining analysis revealed that the levels of 3 potent angiogenic inhibitors (endostatin, PEDF, and TSP-1) were significantly reduced in Bruch's membrane in eyes with AMD. The 3 molecules are distinctly different from each other in structure, relationship to extracellular matrix, and mechanism of inhibition, yet all are potent inhibitors of angiogenesis. Reduced levels of angiogenic inhibitors may make Bruch's membrane more vulnerable to the invasion of CNV.

Bruch's membrane assumes importance in the physiology of the eye by virtue of its strategic location. It is interposed between the metabolically active photoreceptors and RPE and their major source of nutrition and oxygen, the CC. In addition to acting as a support element and an attachment site for the RPE, Bruch's membrane also provides a semipermeable filtration barrier through which major metabolic exchange takes place and it functions as a physical barrier to the egress of cells and blood vessels from the choroid into the sub-RPE and subretinal spaces. Disruption of or damage to this physical barrier often results in the growth of CNV into the sub-RPE and/or subretinal spaces. The fact that Bruch's membrane has substantial levels of 3 antiangiogenic factors suggests that it is a biochemical barrier as well. There was a significant decline in the levels of all of the 3 factors in Bruch's membrane during AMD, and the magnitude of the decline in the levels of PEDF and TSP-1 in Bruch's membrane paralleled the severity of AMD. The decline with the severity of AMD was not true for the RPE and CC. The difference between the results for these structures may be that PEDF and TSP-1 are matrix bound. With gradual deterioration of Bruch's membrane in AMD, PEDF and TSP-1 may be released from that matrix into the milieu that includes the RPE and CC.

Various proteins that promote the regression of new vessels, including endostatin, PEDF, TSP-1, and others, are localized in the eye. We have focused on only 3 inhibitors, but in a recent review, Folkman[16] discussed the numerous antiangiogenic agents that have been found to date, many of which may be present in the eye. The Angiogenesis Foundation[17] currently lists 28 antiangiogenic molecules that are known, and several are being evaluated for treatment of CNV. It is possible that some of these antiangiogenic molecules exist in the RPE–Bruch's membrane–CC complex as well and prevent CNV invasion, but these molecules have not yet been investigated in this tissue. Viral vector–mediated delivery of antiangiogenic molecules, such as endostatin[18] and PEDF,[19] has successfully diminished CNV in rodent models.[20] Potential clinical applications for PEDF have included systemic administration to prevent ischemia-induced retinopathy in a murine model,[21] and a phase 1 clinical trial using a PEDF vector delivered intraocularly to treat macular degeneration is under way.[22] Intraocular injection of viral vectors that express PEDF or injection of recombinant PEDF suppresses retinal neovascularization or CNV.[23,24]

Pigment epithelium–derived factor, a 50-kDa secreted glycoprotein isolated from RPE cells,[25] was shown to be a potent inhibitor of angiogenesis.[19] It is a multifunctional protein with demonstrable neurotrophic, neuroprotective, gliastatic, antitumorigenic, antiangiogenic, and anti-vasopermeability properties.[26,27] It blocks endothelial cells from forming new vessels by inducing apoptosis,[21] thus inhibiting neovascularization of certain tumors.

Different parts of the PEDF molecule mediate these various activities, and peptides corresponding to these regions retain the corresponding activity of native PEDF.

Thrombospondin 1 is a large (450-kDa) matricellular glycoprotein secreted by many cell types that binds to different matrix proteins and cell-surface receptors.[28] This ability accounts for the multifunctional nature and sometimes contradictory functions of TSP-1. Thrombospondin 1 is both a stimulator and an inhibitor of angiogenesis. These contradictory functions demonstrate its ability to maintain a dynamic balance in the regulation of angiogenesis.[28] Therefore, the potential role of TSP-1 as a therapeutic agent remains uncertain.[29] It inhibits adhesion of vascular endothelial cells[30,31] and cell invasion as well as tube formation by vascular endothelial cells.[32–34] In human eyes, TSP-1 was localized in the RPE layer and Bruch's membrane[12,35,36] and in epiretinal membranes in several diseases.[37] It also plays a role in ocular vascular homeostasis, and its absence contributes to vascular dysfunction associated with diabetes.[38] Thrombospondin 1 knockout mice show increased vascular density during retinal vascular development.[39]

Endostatin, a 20-kDa C-terminal fragment of collagen XVIII, has been identified as an endogenous angiogenesis inhibitor.[18] Collagen XVIII is the core protein of a heparan sulfate proteoglycan in vascular and epithelial basement membranes.[40] Some proteases, such as matrix metalloproteinases, can cleave a proteinase-sensitive hinge so that endostatin can be released from collagen XVIII and become available.[41,42] Marneros et al[43] demonstrated that the extracellular matrix component of collagen XVIII or endostatin is essential for maintenance of the RPE. Aged Col18a1−/− mice show massive accumulation of electron-dense amorphous material with membranous debris between the RPE and Bruch's membrane that is similar in appearance and composition to basal laminar deposits in early AMD[44] and contains excess basement membrane material. This suggests that the absence of collagen XVIII or endostatin leads to altered properties of Bruch's membrane that either cause the RPE to produce excess basement membrane material or interfere with the clearance of such basement membrane material, eventually resulting in a progressive accumulation of basal laminar deposit–like material under the RPE with age. Endostatin has been shown to inhibit the angiogenic activities[45,46] and known to up-regulate anti-angiogenesis genes; therefore, it is likely to also be an endogenous inhibitor of CNV.[47] Intravitreous or intravenous delivery of endostatin by viral vectors was shown to inhibit diabetic retinopathy and CNV in experimental studies.[48,49]

Whatever the initial stimulus for CNV formation and invasion, it is clear that angiogenic factors are involved, as CNV membrane and RPE cells have been shown to be immunoreactive for various angiogenic factors. All of the aforementioned antiangiogenic factors (endostatin, PEDF, and TSP-1) seem to have well-defined roles in inhibiting the angiogenic activities; therefore, all of the 3 are likely to be endogenous inhibitors of angiogenesis in the choroid. In this study, expression of 3 potent inhibitors decreased in Bruch's membrane with severity of AMD. These data support the hypothesis that the balance between angiogenic and antiangiogenic factors may be altered in AMD by declining levels of inhibitors. We recently observed no significant difference in relative vascular endothelial growth factor immunoreactivity levels in the RPE–Bruch's membrane–CC complex between aged subjects and subjects with AMD,[11] suggesting that a decline in

antiangiogenic agents and not an increase in angiogenic factors may upset the balance that is normally present. Recent studies also have clarified the importance of a balance between local inhibitory and stimulatory factors in pathological angiogenesis.[50] When there is a shift toward more of the positive regulators and/or less of the negative regulators, this likely would favor a proangiogenesis state leading to the progression of pathological disorders associated with excessive angiogenesis. In the case of AMD, the antiangiogenic barrier of Bruch's membrane is compromised.

CONCLUSIONS

We conclude that the potent endogenous angiogenesis inhibitors are constitutively localized in the RPE–Bruch's membrane–CC complex and may provide a biochemical barrier to prevent CNV formation and progression. The significantly reduced levels of 3 endogenous angiogenesis inhibitors in Bruch's membrane of eyes with AMD may make Bruch's membrane vulnerable to invasion by CNV. These angiogenesis inhibitors may have distinct mechanisms of action or molecular targets, but all are associated with Bruch's membrane. Combinations of 2 or more angiogenic inhibitors with different molecular mechanisms or targets may achieve synergistic effects on CNV as demonstrated recently for retinal neovascularization.[51] Decreased levels of endogenous angiogenesis inhibitors at the Bruch's membrane–RPE complex in the macular region of eyes with AMD have not only opened a new field for investigation into the pathogenesis of CNV but also revealed a new target for pharmacological interventions.

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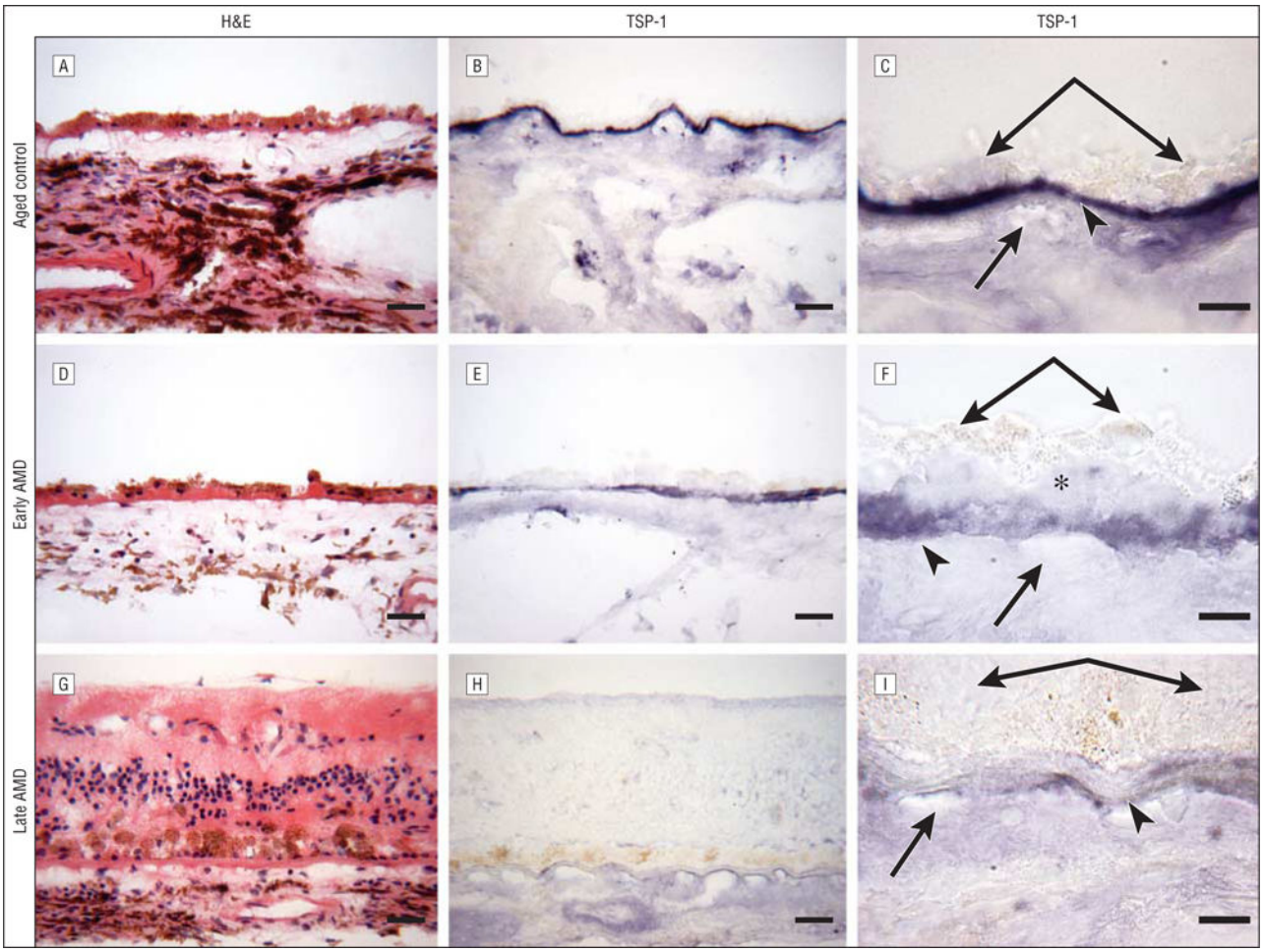

Figure 1.

Immunostaining for thrombospondin 1 (TSP-1). Submacular choroid from a healthy aged control eye (subject 4) (A–C), an eye with early age-related macular degeneration (AMD) (subject 10) (D–F), and an eye with late AMD (subject 19) (G–I). A, D, and G, Hematoxylin and eosin (H&E) staining shows morphological features of the choroid. B and C, In the aged control eye, TSP-1 immunoreactivity is intense in Bruch's membrane. In Bruch's membrane, TSP-1 immunoreactivity is weaker in the eye with early AMD (E and F) compared with the aged control eye (B and C) and is greatly reduced in the eye with late AMD (H and I) compared with both the aged control eye (B and C) and the eye with early AMD (E and F). C, F, and I are high-magnification photographs of B, E, and H, respectively. Double arrows indicate retinal pigment epithelium; arrowheads, Bruch's membrane; single arrows, choriocapillaris; and asterisk, basal laminar deposits. Bars indicate 30 μm in A, B, D, E, G, and H and 10 μm in C, F, and I.

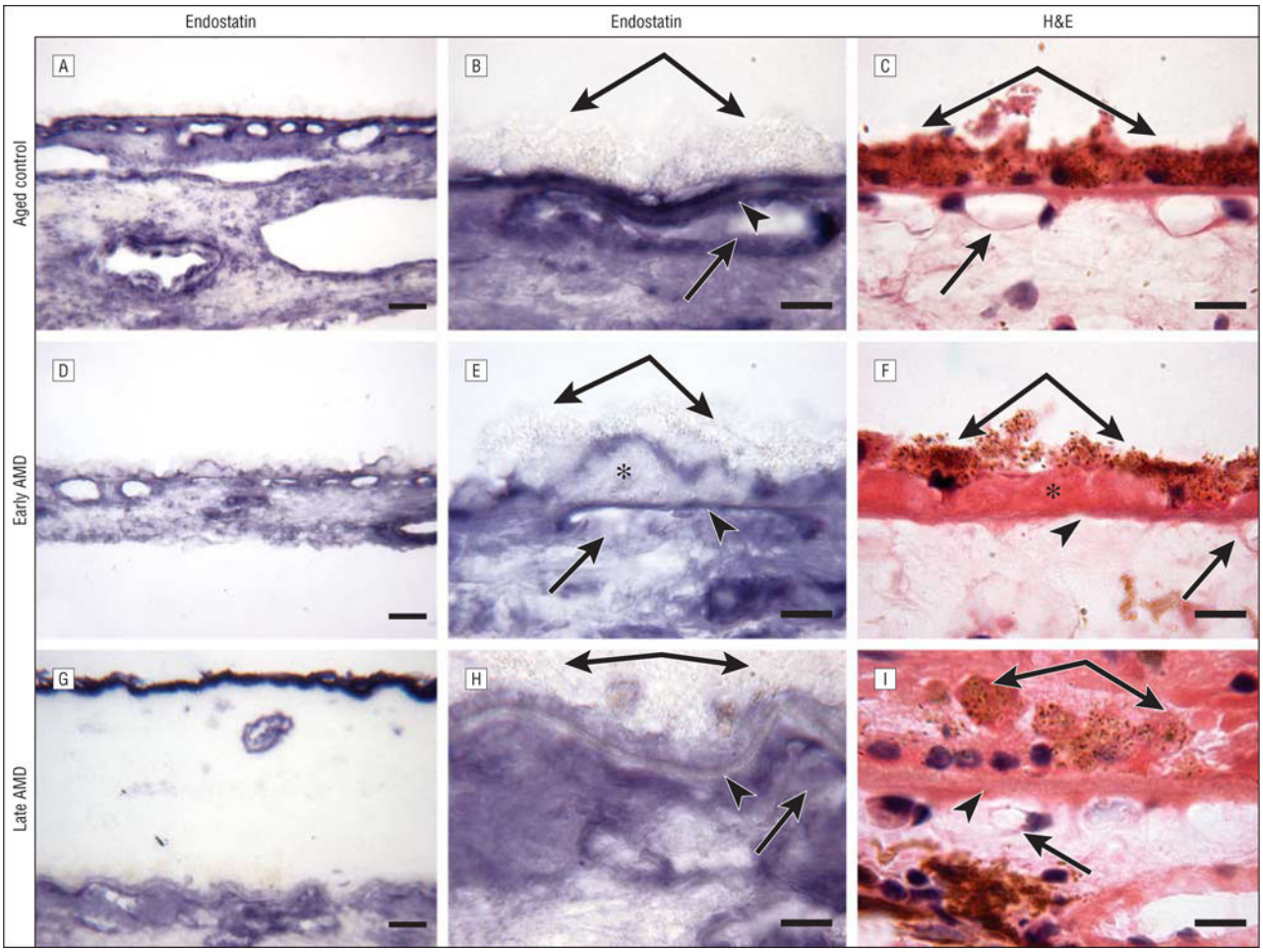

Figure 2.

Immunostaining for endostatin. An aged control eye (subject 4) (A–C), an eye with early age-related macular degeneration (AMD) (subject 10) (D–F), and an eye with late AMD (subject 19) (G–I). C, F, and I, Hematoxylin and eosin (H&E) staining demonstrates the morphological changes in the retina and choroid, such as migration of retinal pigment epithelial cells into the retina in late AMD (I). F, Eosin stains the basal laminar deposits intensely pink in the eye with early AMD. A and B, In the aged control eye, endostatin is prominent in the retinal pigment epithelium basal lamina, Bruch's membrane, and choriocapillaris. The pattern of immunostaining of endostatin appeared similar between the eye with early AMD (D and E) and the aged control eye (A and B), but the endostatin immunoreactivity in Bruch's membrane is weaker in the eye with early AMD (D and E) compared with the aged control eye (A and B). The expression of endostatin is greatly reduced in Bruch's membrane in the eye with late AMD (G and H) compared with the aged control eye (A and B), and the reaction product of endostatin appears more diffuse in the choroidal stroma. B, E, and H are high-magnification photographs of A, D, and G, respectively. Double arrows indicate retinal pigment epithelium; arrowheads, Bruch's membrane; single arrows, choriocapillaris; and asterisks, basal laminar deposits. Bars indicate 30 μm in A, D, and G and 10 μm in B, C, E, F, H, and I.

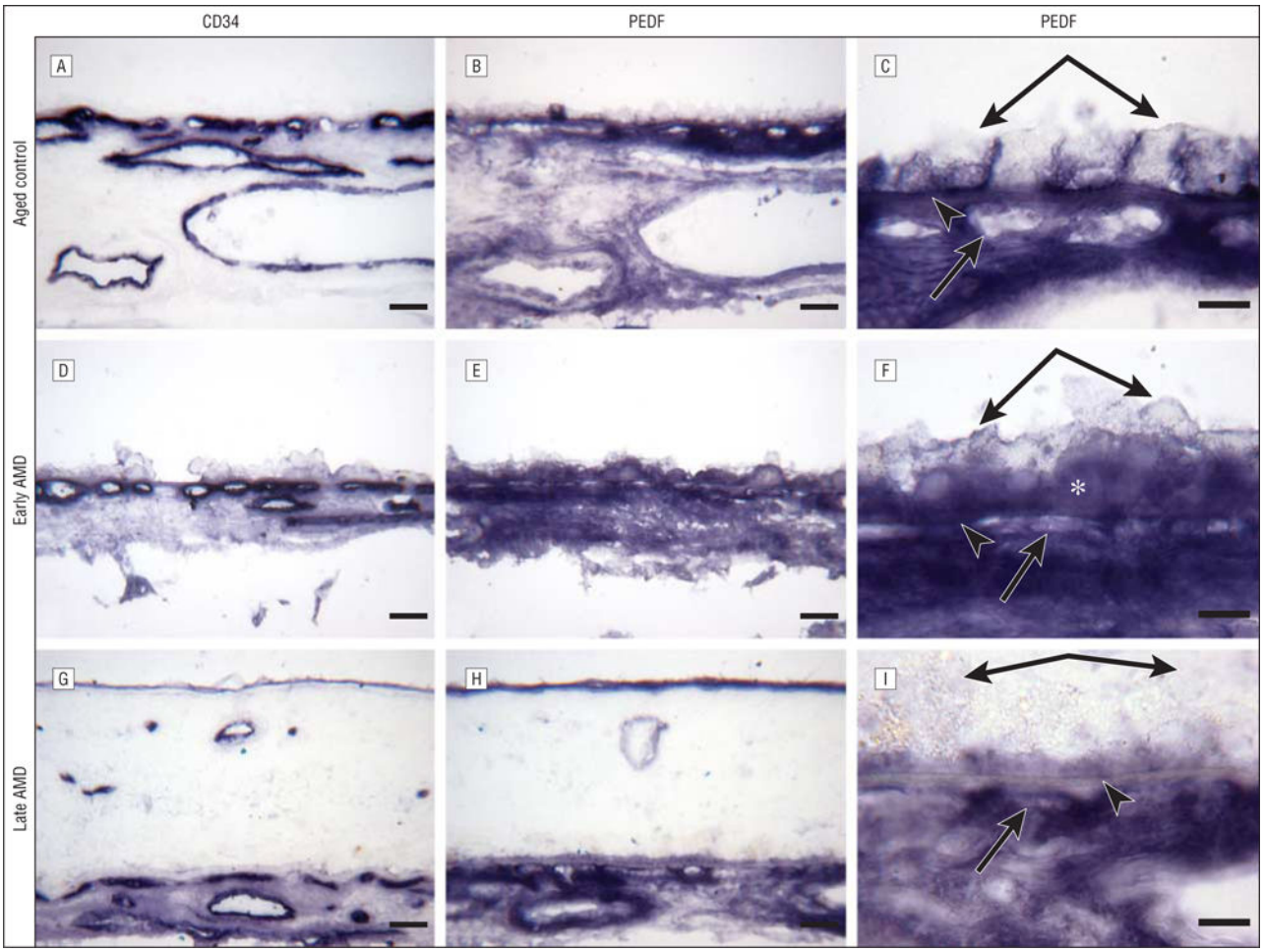

Figure 3.

Immunostaining for pigment epithelium–derived factor (PEDF). Frozen sections of choroid from an aged control eye (subject 4) (A–C) and an eye with early age-related macular degeneration (AMD) (subject 10) (D–F) and of a retina and choroid from a subject with late AMD (subject 19) (G–I). A, D, and G, Staining with CD34 is associated with the choroidal blood vessels. The choriocapillaris appears normal by CD34 localization in the aged control eye (A) and the eye with early AMD (D), whereas CD34 localization demonstrates a highly constricted choriocapillaris in some areas with some lumens not positive for CD34, suggesting no viable endothelial cells, in the eye with late AMD (G). B and C, Pigment epithelium–derived factor intensely stains the retinal pigment epithelium basal lamina, Bruch's membrane, and choriocapillaris in the aged control eye. In the eye with early AMD (E and F), the pattern of immunostaining of PEDF appears similar to that in the aged control eye (B and C), and the reaction product is more diffuse in the choroidal stroma. F, Note that the basal laminar deposits (asterisk) are labeled with PEDF antibody. In the eye with late AMD (H and I), PEDF immunoreactivity is prominent but weaker in Bruch's membrane compared with that in the aged control eye (B and C) and the eye with early AMD (E and F). C, F, and I are high-magnification photographs of B, E, and H, respectively. Double arrows indicate retinal pigment epithelium; arrowheads, Bruch's membrane; and single arrows, choriocapillaris. Bars indicate 30 μm in A, B, D, E, G, and H and 10 μm in C, F, and I.

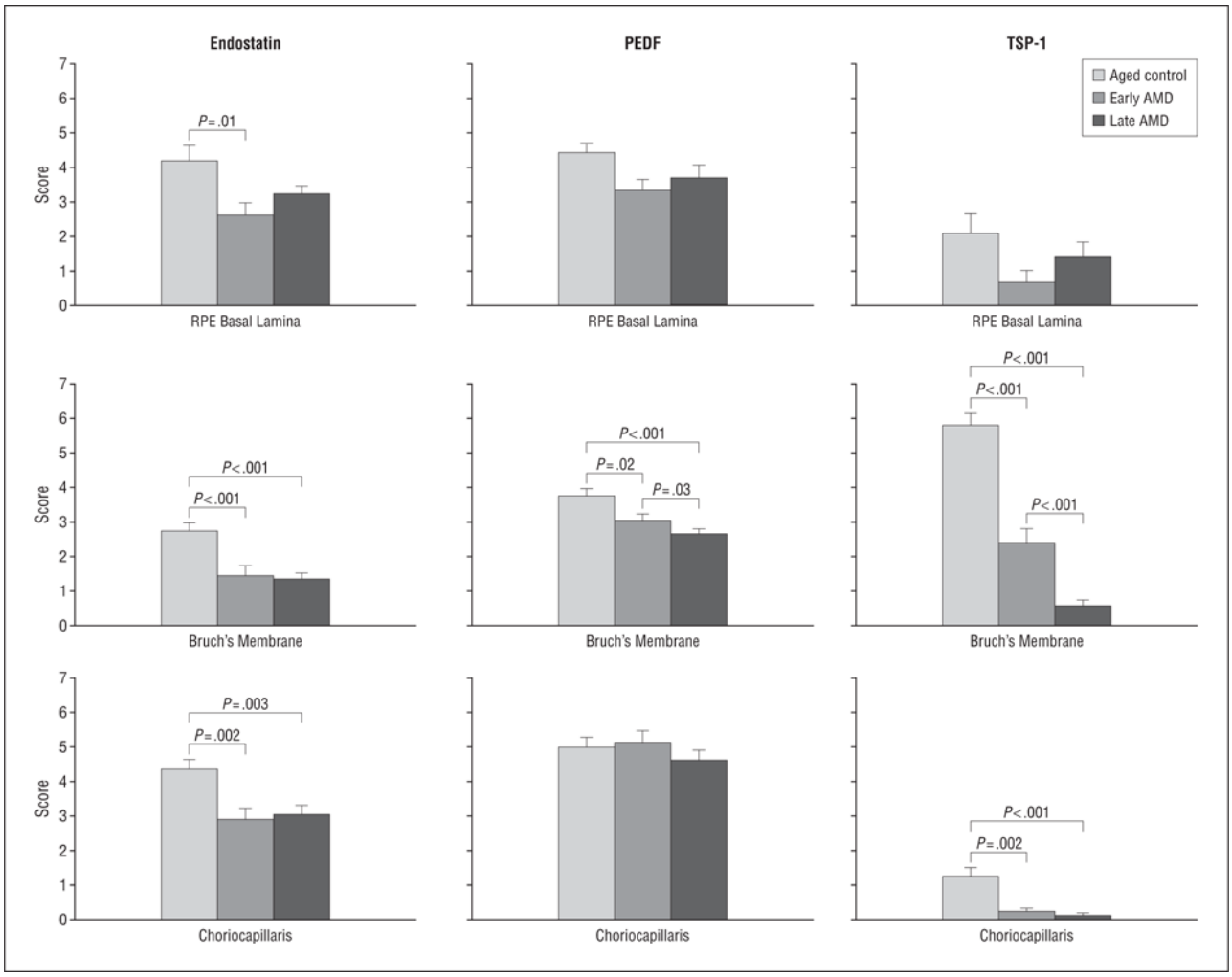

Figure 4.

The mean scores of staining with endostatin, pigment epithelium–derived factor (PEDF), and thrombospondin 1 (TSP-1) between aged control eyes, eyes with early age-related macular degeneration (AMD), and eyes with late AMD. The significance of the difference between the groups by *t* test is indicated. RPE indicates retinal pigment epithelium; error bars, standard deviation.

Table 1

Characteristics of Human Donor Cryopreserved Eyes

Subject No./Sex/Age, y[a]	Time, h		Primary Cause of Death	Medical History	Ocular Diagnosis
	Death to Enucleation	Death to Fixation			
1/F/75	2.5	33	Heart disease		Healthy
2/F/76	7	27	Lung cancer	HTN	Healthy
3/M77	1	26	COPD	HTN	Healthy
4/M/80	2.5	28	COPD		Healthy
5/M/80	7.15	28	Intracranial hemorrhage	HTN	Healthy
6/M/82	3	15	Metastasis brain cancer		Healthy
7/M/83	3	16	Cardiac respiratory arrest		Healthy
8/F/86	5	26	Respiratory failure		Healthy
9/M/61	3.5	34	Metastasis esophageal cancer		AMD, early
10/M/74	4	33	Prostate cancer		AMD, early
11/M/79	3	33	Pneumonia	HTN, asthma	AMD, early
12/F/81	5	29	Myocardial infarction	HTN	AMD, early
13/M/83	3	12	Prostate cancer	DM, HTN	AMD, early
14/F/98	2	33	Old age		AMD, early
15/F/69	3.5	40	Subarachnoid hemorrhage	Pulmonary fibrosis	AMD (GA), late
16/M/75	7	30	Aspiration pneumonia		AMD (GA), late
17/F/93	4	20	Multisystem failure	DM, HTN	AMD (disciform scar), late
18/M/94	3	36	Cardiac failure		AMD (disciform scar), late
19/M/95	3.5	Unknown	Cardiomyopathy		AMD (disciform scar), late
20/M/105	4.5	11	COPD		AMD (disciform scar, GA), late

Abbreviations: AMD, age-related macular degeneration; COPD, chronic obstructive pulmonary disease; DM, diabetes mellitus; GA, geographic atrophy; HTN, hypertension.

[a]All of the donors were white.

Table 2

Comparison of the Retinal Pigment Epithelium–Bruch's Membrane–Choriocapillaris Complex Parameters in 8 Aged Control Eyes, 6 Eyes With Early Age-Related Macular Degeneration, and 6 Eyes With Late Age-Related Macular Degeneration

		Immunoreactivity Scores, Mean (SD)			
Factor	**Parameter**	**Aged Control Eyes**	**Eyes With Early AMD**	**Eyes With Late AMD**	***P* Value[a]**
Endostatin	Bruch's membrane	2.750 (1.0)	1.444 (1.1)	1.333 (0.7)	.01
	RPE basal lamina	4.188 (2.1)	2.611 (1.5)	3.222 (1.0)	.19
	CC	4.354 (1.4)	2.889 (1.4)	3.028 (1.2)	.06
PEDF	Bruch's membrane	3.875 (0.9)	3.167 (0.8)	2.556 (0.7)	.01
	RPE basal lamina	4.583 (1.4)	3.583 (1.3)	3.556 (1.8)	.36
	CC	5.354 (1.4)	5.333 (1.4)	4.611 (1.1)	.46
TSP-1	Bruch's membrane	5.792 (1.6)	2.389 (1.7)	0.556 (0.7)	< .001
	RPE basal lamina	2.083 (2.7)	0.667 (1.4)	1.389 (1.8)	.51
	CC	1.250 (1.2)	0.222 (0.4)	0.111 (0.3)	.02

Abbreviations: AMD, age-related macular degeneration; CC, choriocapillaris; PEDF, pigment epithelium–derived factor; RPE, retinal pigment epithelium; TSP-1, thrombospondin 1.

[a] *P* values from 1-way analysis of variance.