Invest Ophthalmol Vis Sci. Author manuscript; available in PMC 2014 March 09.

Published in final edited form as:
Invest Ophthalmol Vis Sci. 2008 February ; 49(2): 743–750. doi:10.1167/iovs.07-1072.

Complement-Associated Deposits in the Human Retina

Aditya Seth, **Jing Cui**, **Eleanor To**, **Melissa Kwee**, and **Joanne Matsubara**
Department of Ophthalmology and Visual Sciences, University of British Columbia, Vancouver, British Columbia, Canada

Abstract

Purpose—The purpose of this study was to investigate complement activation and associated inflammatory mechanisms in normal, aged human retina.

Methods—Evidence of complement activation and associated mechanisms was assessed in normal human retina ($n = 52$) using a panel of antibodies directed against membrane attack complex (C5b-9), microglia (CD11b), amyloid precursor protein (APP), scavenger receptor (CD36), and a phytolectin (RCA-I). Fifty-two eyes, categorized into two age groups, were used. Nineteen “younger” eyes (<56 years) and 33 “older” eyes (>69 years) with no history of ocular disease were processed between 4 and 22 hours, with a median delay of 14 hours postmortem.

Results—Age-dependent expression was evident in C5b-9, APP, CD11b, and RCA-I, but not CD36, immunoreactivity. Immuno-reactivity for C5b-9 was robust in Bruch membrane (BM) and the intercapillary pillars of Bruch. Immunoreactivity for APP was robust in the basal cytoplasm of the retinal pigment epithelium. Immunoreactivity for CD11b was robust on the surface of the retinal pigment epithelial cell, in the choriocapillaris, and in BM. Lectin binding of RCA-I was strong throughout the neuroretina.

Conclusions—Robust immunostaining for APP in older donor eyes suggested that amyloid beta peptides may be one of the triggers of complement activation during the normal aging process. Microglial markers CD11b and RCA-I also increase with age, suggesting a concomitant inflammatory response to C5b-9 deposits in the retinal pigment epithelium, BM, and CC. Immunoreactivity for CD36 was strong in both age groups; the lack of age dependence in this candidate receptor for amyloid beta suggested that complement activation may arise from interactions of amyloid beta with other candidate receptors in normal human retina.

Recent studies have provided novel insight into the role of the complement system and inflammation in the pathogenesis of AMD. Several groups have identified a polymorphism in the complement factor H (*CFH*) gene that is linked to an increased risk for AMD.[1–4] Complement factor H is an inhibitory regulator of the “alternative” pathway of the complement system. Mutations in this gene have been suggested to cause uncontrolled complement activation, leading to chronic inflammation in the retina.

Corresponding author: Joanne Matsubara, Department of Ophthalmology and Visual Sciences, Eye Care Centre, 2550 Willow Street, Vancouver BC V5Z 3N9, Canada; jms@interchange.ubc.ca.

Disclosure: **A. Seth**, None; **J. Cui**, None; **E. To**, None; **M. Kwee**, None; **J. Matsubara**, None

Although the complement system has been implicated in the pathogenesis of AMD, it is not known what causes or triggers the complement system to go awry in AMD. At present, it is thought that several candidate molecules may activate the complement system in AMD. Amyloid beta is a constituent of drusen, the hallmark extracellular deposits in the dry form of AMD, and is a known activator of the complement system.[5,6] Amyloid beta deposits in drusen, like the amyloid beta deposits in senile plaques of Alzheimer disease, are associated with activated complement proteins and cell injury.[7–10] Retinal pigment epithelial (RPE) cells and retinal neurons express amyloid precursor protein (APP) and may provide a local retinal source of amyloid peptides.[11,12] An earlier study reported age-related changes in amyloid beta deposits in the normal human retina, which, if substantiated, would support the hypothesis that AMD may be associated with age-related cellular changes that promote complement activation in the retina.[11]

Dysregulation of the receptor for the candidate activators of the complement cascade may also promote inflammation in the retina. The class B scavenger receptor CD36 recognizes and binds to amyloid beta and other ligands that play a role in triggering the immune response.[13–15] CD36 is expressed on several types of cells in the retina, including retinal pigment epithelium, where it is involved with the phagocytosis of rod outer segments.[16–18] This receptor is also present on micro-vascular endothelial, macrophage, and microglial cells and has been implicated in a number of diseases, including atherosclerosis, cancer, diabetes, cardiovascular disease, and Alzheimer disease.[19–21] Cellular expression of CD36 aids in cellular homeostasis by the clearance of aberrant lipoproteins and confers protection against pathogens and inflammation. An age-dependent loss of CD36 receptors may result in abnormal RPE function, which may then directly or indirectly promote the complement cascade (Houssier M, et al. *IOVS* 2007;48:ARVO E-Abstract 25).

Retinal damage caused by the complement cascade may also originate from the recruitment of macrophages and microglia cells. These cells attempt to minimize retinal inflammation, but they also cause “bystander” damage by releasing reactive oxygen species. Reactive oxygen species promote oxidative injury to retinal cells, including the RPE cell. It is well known that oxidative stress is an important risk factor in AMD, and it has been shown in a 10-year clinical study sponsored by the National Eye Institute that high-dose antioxidant supplements significantly reduce the risk for advanced AMD by approximately 25%.[22,23] Other studies have shown that oxidative injury to normal retinal tissues may promote the progression of AMD by initiating an abnormal angiogenic response and a wound-healing response causing choroidal neovascularization, a characteristic of the exudative (wet) form of AMD.[24,25]

Drusen from patients with AMD and healthy donors have complement-associated proteins.[8,26,27] To date, the role of complement has been principally studied in diseased eyes, and nothing is known of the changes in complement activation resulting from normal aging in the retina. Because the major risk factor for AMD is increasing age,[25] the present study focuses on age-related changes in complement proteins—candidate triggers of the complement cascade—and microglia in the normal human retina.

Materials and Methods

Eye Tissues

Human donor eyes were obtained from the Eye Bank of British Columbia (Vancouver, BC, Canada). Methods for securing human tissue were humane and included proper written informed consent, in compliance with the Declaration of Helsinki. The procedures were approved by the Clinical Research Ethics Board of the University of British Columbia (Vancouver, BC, Canada). All tissue samples included in this study were measured against criteria that excluded tissues from donors with any of the following four factors: evidence of systemic or local infection; progressive central nervous system disease or systemic disease of unknown etiology; lymphoproliferative or myeloproliferative disorders; intrinsic eye disease or previous ocular surgery.

Retinal tissues were processed within 4 to 22 hours of death (median time, 14 hours). Donor eyes were divided into two age groups, a “younger” group of donors 56 and younger and an “older” group of donors 69 and older. A summary of donor characteristics, including age, sex, and cause of death, is presented (see Table 2).

Tissue Preparation

A 0.5-cm incision was made posterior to the limbus, and the anterior segment of the eye was removed. For frozen preparation, the posterior segment was embedded in molds with OCT embedding medium (Tissue-Tek, Torrance, CA) at −20°C. The embedded tissue was then frozen in liquid nitrogen and stored at −80°C until sectioning. Frozen tissue was serially sectioned at 6 μm with a cryostat (Frigocut 2800 n; Reichert-Jung, Chicago, IL) at −20°C and was mounted onto frosted glass slides, then stored at −20°C until the time of processing. For paraffin embedding, whole globes were fixed by immersion in 10% buffered formalin for 7 days. The eyes were then washed extensively in phosphate-buffered saline (PBS) at pH 7.4, embedded in paraffin, and oriented in the sagittal plane to obtain 6-μm sections through the axis defined by the pupil and optic nerve. Parafoveal sections were selected for the study.

Immunohistochemistry

A summary of the proteins studied and antibodies used in this study is shown in Table 1.

C5b-9 and CD36

Cryosections were air dried for 20 minutes at room temperature and were fixed with 4% paraformaldehyde for 10 minutes. This was followed by antigen retrieval in protease (Proteinase K, 20 μg/mL, pH 8.0; Sigma Aldrich, St. Louis, MO) for 10 minutes at room temperature. To remove endogenous peroxidases, tissues were treated at room temperature with 0.3% H_2O_2 diluted in PBS for 15 minutes and then with 5% normal horse serum in 0.3% solution (Triton X-1000 [TX-100]; Sigma Aldrich) PBS for 20 minutes to reduce nonspecific staining. The sections were then incubated for 2 hours at room temperature and thereafter at 4°C for 48 to 72 hours with either of the following primary antibodies: monoclonal mouse anti– human C5b-9 clone aE11 (1:100; Dako, Glostrup, Denmark), monoclonal mouse anti–human CD36 (1:100; Chemicon, Temecula, CA). Negative control samples were obtained by omitting the primary antibody and incubating selected sections in

the diluent containing TX-100 and normal, nonimmune serum. After incubation in the primary antibody, sections were washed in PBS and subsequently incubated for 30 minutes in a solution containing the appropriate biotinylated secondary antibody (1:100; Vector Laboratories, Burlingame, CA) at room temperature. Next, a standard avidin-biotin-complex-alkaline phosphatase detection system (ABC-AP; Vector Laboratories) was used to incubate sections for 30 minutes at room temperature. After three washes, the sections were developed for 1 hour at room temperature with levamisole alkaline phosphatase substrate solution (Vector Blue; Vector Laboratories) resulting in a bright blue reaction product. Sections were counterstained (Nuclear Fast Red; Vector Laboratories) for 15 minutes and mounted (Crystal Mount; Biomeda, Foster City, CA).

Amyloid Precursor Protein

APP immunohistochemistry was performed in paraffin-embedded tissue. Using the ABC detection system, staining was performed on an automated stainer (Ventana ES; Ventana Medical Systems, Tucson, AZ). Briefly, 6-μm paraffin sections were deparaffinized and rehydrated. Antigen retrieval was performed in 10 mM sodium citrate buffer at high heat in a microwave oven for 13 to 16 minutes. The sections were then pretreated with 0.3% H_2O_2 diluted in PBS to eliminate endogenous peroxidase activity and with 1% bovine serum in PBS for 15 minutes to reduce the nonspecific binding. Next, sections were incubated for 32 minutes with monoclonal mouse anti–APP A4 antibody clone 22C11 diluted in PBS (1:5000; Chemicon). The primary antibody was linked to an avidin-biotin conjugated secondary antibody and was developed with an amino ethyl carbazole chromogen (AEC; Sigma Aldrich) for 6 minutes. The resultant positive stain was red and could be easily distinguished from the endogenous brown pigment in RPE cells. All sections were counterstained with hematoxylin. Positive and negative controls were processed using human brain tissue with Alzheimer disease. Negative control tissue was processed in an identical manner, but the initial incubation was in a solution containing diluent only (nonimmune serum in PBS).

CD11b

CD11b (Mac-1) immunohistochemistry was undertaken in paraffin sections. Sections were deparaffinized, rehydrated, and washed in PBS. Antigen retrieval in protease (Proteinase K; 20 μg/mL, pH 8.0) was undertaken for 10 minutes at room temperature. The tissue was treated at room temperature with 0.3% H_2O_2 diluted in PBS for 15 minutes to remove endogenous peroxidase. Next, sections were incubated in 3% normal horse serum in 0.3% TX-100 PBS for 20 minutes to reduce nonspecific binding. Sections were then incubated for 2 hours at room temperature and thereafter at 4°C for 24 to 48 hours with mouse monoclonal antibody to the human CD11b (Mac-1) antigen (1:600; Invitrogen, Carlsbad, CA). Control sections were incubated in a solution that contained nonimmune serum and TX-100 in PBS (e.g., primary antibody omitted). After they were washed in PBS, sections were incubated in a biotinylated secondary antibody for 30 minutes at room temperature and rinsed. The sections were then incubated in a solution of ABC-AP for 30 minutes at room temperature. After three washes, the sections were developed for 1 hour at room temperature with Vector Blue. Sections were counterstained with Nuclear Fast Red for approximately 15 minutes and mounted (Crystal Mount; Biomeda).

RCA-I Lectin Binding

The plant lectin *Ricinus communis* agglutinin I (RCA-I) was used to mark resting and activated microglia cells in frozen sections of tissue.[28] Cryosections were air dried for 20 minutes and fixed with 4% paraformaldehyde for 10 minutes at room temperature. The sections were pretreated with 0.3% H_2O_2 for 20 to 30 minutes to eliminate endogenous peroxidase activity and subsequently with 1% bovine serum albumin in PBS for 30 minutes to reduce nonspecific binding. After they were rinsed with PBS, the sections were incubated in biotinylated RCA-I (1:1000; Vector Laboratories) diluted in PBS with 0.3% TX-100 for 1 hour at room temperature and then overnight at 4°C. After rinsing, the sections were incubated with ABC-AP for 30 minutes at room temperature, followed by incubation in vector blue. Sections were counterstained with nuclear fast red for 1 minute, dehydrated, and coverslipped with vector mount (Vector Laboratories). Negative controls were prepared identically with the omission of the biotinylated lectin solution.

For each of the five markers used in this study, tissue sections (five per donor) from both age groups were reacted simultaneously and processed in an identical manner. Tissue sections from all staining procedures were examined and photographed at 60× or 100× magnification under a light microscope (Eclipse 80i; Nikon, Tokyo, Japan) with a digital camera (CoolPix 5400; Nikon).

Statistical Analysis

Immunohistochemical results from the RPE and choroidal complex were analyzed in a masked fashion and presented here. Semiquantitative analysis was conducted by examination of five fields per tissue section using a 40× objective lens and 10× eyepieces. Four to five tissue sections per donor were analyzed for each marker. Immunostaining was graded based on the relative intensity of immunostaining for each marker. In some cases, such as for APP, the number of RPE cells demonstrating immunoreactivity was counted, and an average for all fields per tissue section was determined.[29,30] Robust immunostaining intensity (or >10 APP immunoreactive cells per field) was classified as+++, indicating strong expression. Moderate immunoreactivity intensity (or 6–10 APP immunoreactive cells per field) was classified as ++, and a field with sparse immunoreactivity (or 1–5 APP immunoreactive cells per field) was classified at +, indicating mild expression. No expression above background was signified as −. The intensity of staining for each donor was assigned a value based on this semiquantitative analysis (Table 2). Student's *t*-test (unequal variance) was used for statistical analysis of immunohistochemical data from the two age groups. A one-tailed test was selected based on the directional hypothesis that complement-associated deposits in the retinal pigment epithelium and choroid increase with age. Significance level was set at $P < 0.05$.

Results

Results of studies on C5b-9, CD11b, RCA-I binding CD36, and APP immunoreactivity were based on the analysis of 52 normal eyes. Nineteen eyes were from donors 56 years of age or younger (11 women, 8 men). The other 33 eyes were obtained from donors 69 years of age or older (8 women, 25 men).

C5b-9

C5b-9 immunoreactivity was assessed in the RPE–choroidal complex of younger and older donor eyes. Immunoreactivity was strongest in Bruch membrane and the intercapillary pillars of Bruch in tissues from older donors. The RPE monolayer was not immunoreactive for C5b-9, though faint immunoreactivity in the basal infoldings of some RPE cells was observed at high power (Fig. 1A, inset). Drusen were present in the eyes of donors in the older group, though the donors had not been known to have symptoms of AMD. Drusen sites, when present, were strongly immunoreactive for C5b-9 (Figs. 1C, 1D).

In the older donor eye group, 11 of 15 (73%) eyes were positive for C5b-9. Three eyes exhibited strong (+++) immunoreactive intensity, whereas eight eyes exhibited mild (+) to moderate (++) immunoreactive intensity for C5b-9. In the younger donor eye group, only two of 15 eyes (13%) exhibited C5b-9 immunoreactivity of moderate (++) intensity. None of the 15 eyes from donors 56 or younger exhibited C5b-9 staining of strong (+++) intensity. Statistical analysis of C5b-9 immunoreactivity reached significance between the two age groups (Table 2).

CD11b

CD11b (Mac-1) immunoreactivity targets activated microglial populations.[31–34] CD11b immunoreactivity was robust on the apical surfaces of a subset of RPE cells (Figs. 1E, 1G) in perivascular cells in the choriocapillaris (Fig. 1H) and to a lesser extent on Bruch membrane (Fig. 1E). A subset of RPE cells remained negative for CD11b (Fig. 1F). Tissue samples from both age groups demonstrated CD11b immunoreactivity. Eight of 11 eyes from the older donor eye group and three of four of the samples from the younger group were positive for CD11b immunoreactivity. The eight positive samples from the older donor eye group scored in the moderate (++) to strong (+++) immunoreactivity categories. In the younger donor eye group, the three positive samples scored in the mild (+) and moderate (++) categories. Statistical analysis of CD11b immunoreactivity in eye tissues of older compared with younger donor eyes reached significance (Table 2).

RCA-I

RCA-I staining was observed throughout the retinal layers but was strongest in the outer plexiform layer and at the border between the outer nuclear layer and the outer segments of the photoreceptors (Fig. 1I). In the tissue samples from the younger donor eye group, RCA-I staining revealed three of 14 eyes (21%) positive compared with 11 of 12 eyes (91%) positive in the older group. In the younger donor eye group, only moderate staining (++) was observed, whereas moderate staining and strong staining were observed in the older donor eye group. Statistical analysis reached significance ($P < 0.05$), indicating immunoreactivity for RCA-I was stronger in the older than in the younger donor eye group (Table 2).

APP

APP immunoreactivity was assessed in the RPE–choroidal complex of younger and older donor eye groups. Immunoreactivity was present in the cytoplasm of the basal compartment of a subset of RPE cells (Figs. 1J, 1K). In the younger donor eye group, only one of three eyes exhibited weak (+) APP immunoreactivity. In the older donor eye group, six of seven

eyes expressed APP immunoreactivity. Of these six eyes, three exhibited strong (+++) immunostaining for APP. Statistical analysis reached significance ($P < 0.05$; Table 2).

CD36

CD36 immunoreactivity was present on the RPE cells of younger and older donor eye samples. Strong immunoreactivity was seen in the RPE cell, Bruch membrane, intercapillary pillars of Bruch, and basal deposits beneath RPE cells when present (Fig. 1L, M). Ten of 12 (83%) samples from the younger donor eye group were positive for CD36 immunoreactivity. In the older donor eye group, tissue from all 14 (100%) eyes demonstrated positive CD36 immunoreactivity. Statistical analysis of CD36 immunoreactivity in eye tissues of older compared with younger donor eyes did not reach significance ($P = 0.11$) (Table 2).

Discussion

The present study addresses the age-dependent deposition of complement proteins, inflammation, and candidate activators of the complement cascade in the RPE–choroidal complex of the healthy adult donor eye. An important limitation of this study is the use of postmortem tissue and the time delay between death of donor and fixation of eye tissue. In this study, the postmortem time interval ranged between 4 and 22 hours, with a median delay of 14 hours. Wu et al.[35] reported that immunoreactivity for glial fibrillary acidic protein in human retina was significantly affected only after prolonged (e.g., >30 hours) postmortem times. Although the tissues in the present study did not reach 30 hours of postmortem delay, we are cognizant of the variability in immunoreactivity that might have occurred because of postmortem delay; we designed the study with a large sample size to compensate in part for the technical challenges associated with donor tissue.

A summary of the findings is depicted in Figure 2. Our results demonstrate that the immunoreactivity for the terminal product of the complement cascade, C5b-9, was significantly stronger in Bruch membrane and the intercapillary pillars of Bruch of the eye tissues in the older compared with the younger donor eyes. Because the eye tissues used in this study was from donors with no history of eye disease, these results suggest that age plays a role in activating the complement cascade in the normal retina. An increase in C5b-9 deposits in normal aged retina may be one of the underlying reasons age has been identified as the major risk factor for AMD.[3,27] Clearly, genetic and environmental factors are also involved in the pathogenesis of AMD. However, the results of the present study reemphasize that the normal aging process contributes to the activation of complement system in the outer retina. Complement activation is likely a part of the normal aging process because other tissues, such as brain, also demonstrate an age-dependent activation of the complement pathways.[36]

In a study on diabetic retinopathy and complement activation, ancillary evidence for C5b-9 immunoreactivity in Bruch membrane and the intercapillary pillars of Bruch in two older, normal donor eyes was presented in Gerl et al.,[37] corroborating our findings. Complement cofactor protein (CD46), a regulatory protein that inactivates C3b, was shown by Vogt et al. [38] to be age independent on human RPE cells. However, the small sample size in Vogt et al.

[38] (n = 8; for ages 47–85 years) might have limited the likelihood of observing any relationship with age.

We also showed that the microglial markers RCA-I and CD11b demonstrated increased expression concomitant with increasing age. RCA-I binds selectively to oligosaccharides on quiescent and activated retinal microglia cells, whereas CD11b is a surface receptor that is sharply upregulated in activated microglia. Our results are consistent with the increase in CD45 immunoreactivity in older compared with younger retinas reported earlier.[39] Microglial cells became activated and migrate toward complement deposits; therefore, their increased frequency in the retinal tissues of the older donor eye group was consistent with stronger C5b-9 immunoreactivity in the age group reported here. Given that microglial cells responded to the deposition of C5b-9 in the outer retina, oxidative injury to RPE cells might have been exaggerated because the reactive oxygen species secondarily produced activated microglia.

The RPE cell has been suggested as a local retinal source for complement activation.[8,40] In this study, we hypothesized that amyloid beta is one of the triggers of the complement cascade in RPE cells. APP immunoreactivity was greater in eye tissues of the older donor eye group than the younger one. This finding is consistent with the study by Loffler et al.,[11] which reported generally stronger immunoreactivity for APP and amyloid beta in the retinal pigment epithelium of older normal eyes. In addition to the RPE cell as a local retinal source of APP, it is known that plasma amyloid beta protein levels increase with age.[41] Because amyloid beta is a known activator of the complement cascade,[42–44] it is conceivable that an age-dependent increase in plasma amyloid levels, in addition to a local retinal source of amyloid beta, may both work toward complement activation. For comparison, the central nervous system also demonstrates an age-dependent increase in amyloid beta deposits in healthy donors, which appears to be further increased in brain tissues from Alzheimer disease.[45,46]

At present, little is known regarding the identity of the receptor(s) that bind amyloid beta in RPE cells. A candidate receptor, the scavenger receptor CD36, was studied here. CD36 immunoreactivity was strong in younger (83%) and older (100%) donor eye groups. Because this receptor is known to bind to modified lipoproteins and peptides such as amyloid beta, it was hypothesized that CD36 immunoreactivity may also increase in the retina with age.[47] However, the results presented here did not support this hypothesis. Interestingly, a recent study reported that CD36 knockout mice have progressive choroidal involution, a major symptom of the nonexudative form of AMD (Houssier M, et al. *IOVS* 2007;48:ARVO E-Abstract 25). Thus, the regulation of CD36 may be associated with factors involving the pathologic changes in the RPE layer and may impact disease progression at a stage further downstream of complement activation.

It is also possible that amyloid beta may interact with other receptors, such as the multiligand receptor for advanced glycation end products (RAGE), also known to be expressed by RPE cells and upregulated in normal aging.[48,49] Activation of RAGE has been shown to initiate a program of inflammatory genes.[50] Future studies to address the role of

amyloid beta stimulation of RPE cells may elucidate the receptor(s) and downstream signaling pathways associated with complement activation in the eye.

Acknowledgments

Supported by Canadian Institutes of Health Research (CIHR) MOP Grant 77736 (JM), a CIHR research grant (JM), a Faculty of Medicine, University of British Columbia, Summer Studentship award (AS), and Helen Keller Foundation for Research and Education (JM).

The authors thank Valerie White for discussions throughout the course of this study; the Eye Bank of British Columbia for access to donor eyes; Pat McGeer for providing a sample of Alzheimer brain tissue for positive controls in the immunohistochemical studies; and Joanne Boetzke and Alice Jang for assisting in the histologic preparation of eye tissues.

References

1. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. Science. 2005; 308:419–421. [PubMed: 15761120]
2. Edwards A, Ritter R 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. Science. 2005; 308:421–424. [PubMed: 15761121]
3. Klein RJ, Zeiss C, Chew Y, et al. Complement factor H polymorphism in age-related macular degeneration. Science. 2005; 308:385–389. [PubMed: 15761122]
4. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. Proc Natl Acad Sci USA. 2005; 102:7227–7232. [PubMed: 15870199]
5. Dentchev T, Milam AH, Lee VMY, Trojanowski JQ, Dunaief JL. Amyloid beta is found in drusen from some age-related macular degeneration retinas, but not in drusen from normal retinas. Mol Vis. 2003; 9:184–190. [PubMed: 12764254]
6. Anderson DH, Talaga C, Rivest AJ, Barron E, Hageman GS, Johnson LV. Characterization of amyloid assemblies in drusen: the deposits associated with aging and age-related macular degeneration. Exp Eye Res. 2004; 78:243–256. [PubMed: 14729357]
7. Johnson LV, Ozaki S, Staples MK, Erickson PA, Anderson DH. A potential role for immune complex pathogenesis in drusen formation. Exp Eye Res. 2000; 70:441–449. [PubMed: 10865992]
8. Hageman GS, Luthert PJ, Chong NHV, Johnson LV, Anderson DH, Mullins RF. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. Prog Ret Eye Res. 2001; 20:705–732.
9. Anderson DH, Mullins RF, Hageman GS, Johnson LV. A role of local inflammation in the formation of drusen in the aging eye. Am J Ophthalmol. 2002; 134:411–431. [PubMed: 12208254]
10. Luibl V, Isas JM, Kayed R, Glabe G, Langen R, Chen J. Drusen deposits associated with aging and age-related macular degeneration contain nonfibrillar amyloid oligomers. J Clin Invest. 2006; 116:378–385. [PubMed: 16453022]
11. Loffler KU, Edward DP, Tso MOM. Immunoreactivity against Tau, amyloid precursor protein, and beta-amyloid in the human retina. Invest Ophthalmol Vis Sci. 1995; 36:24–31. [PubMed: 7822152]
12. Johnson LV, Leitner WP, Rivest AJ, Staples MK, Radeke MJ, Anderson DH. The Alzheimer's A-peptide is deposited at sites of complement activation in pathologic deposits associated with aging and age-related macular degeneration. Proc Natl Acad Sci USA. 2002; 99:11830–11835. [PubMed: 12189211]
13. Hoebe K, Georgel P, Rutschmann S, Du X, Mudd S, Crozat K. CD36 is a sensor of diacylglycerides. Nature. 2005; 433:523–527. [PubMed: 15690042]
14. Stuart LM, Deng J, Silver JM, Takahashi K, Tseng AA, Hennessy EJ. Response to *Staphylococcus aureus* requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain. J Cell Biol. 2005; 170:477–485. [PubMed: 16061696]

15. Mwaikambo BP, Sennlaub F, Ong H, Chemtob S, Hardy P. Activation of CD36 inhibits and induces regression of inflammatory corneal neovascularization. Invest Ophthalmol Vis Sci. 2006; 47:4356–4364. [PubMed: 17003426]
16. Ryeom SW, Sparrow JR, Silverstein RL. CD36 participates in the phagocytosis of rod outer segments by retinal pigment epithelium. J Cell Sci. 1996; 109:387–395. [PubMed: 8838662]
17. Finnemann SC, Silverstein RL. Differential roles of CD36 and $\alpha v\beta 5$ integrin in photoreceptor phagocytosis by the retinal pigment epithelium. J Exp Med. 2001; 194:1289–1298. [PubMed: 11696594]
18. Tserentsoodol N, Gordiyenko NV, Pascual I, Lee JW, Fliesler SJ, Rodrigues IR. Intraretinal lipid transport is dependent on high density lipoprotein like particles and class B scavenger receptors. Mol Vis. 2006; 12:1319–1333. [PubMed: 17110915]
19. Febbraio M, Hajjar DP, Silverstein RL. CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. J Clin Invest. 2001; 108:785–791. [PubMed: 11560944]
20. Basta G, Schmidt AM, De Caterina R. Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. Cardiovasc Res. 2004; 63:582–592. [PubMed: 15306213]
21. Febbraio M, Silverstein RL. CD36: implications in cardiovascular disease. Int J Biochem Cell Biol. 2007; 39:2012–2020. [PubMed: 17466567]
22. Age-Related Eye Disease Study Research Group. Risk factors associated with age related macular degeneration: a case-control study in the Age-Related Eye Disease Study. Age-Related Eye Disease Study Report No. 3. Ophthalmology. 2000; 107:2224–2232. [PubMed: 11097601]
23. Age-Related Eye Disease Study Research Group. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss. Age-Related Eye Disease Study Report No. 8. Arch Ophthalmol. 2001; 119:1417–1436. [PubMed: 11594942]
24. Cai J, Nelson KC, Wu M, Sternberg P Jr, Jones DP. Oxidative damage and protection of the RPE. Prog Ret Eye Res. 2000; 19:205–221.
25. Zarbin MA. Current concepts in the pathogenesis of age-related macular degeneration. Arch Ophthalmol. 2004; 122:598–614. [PubMed: 15078679]
26. Crabb JW, Miyagi M, Gu X, et al. Drusen proteome analysis: an approach to the etiology of age related macular degeneration. Proc Natl Acad Sci USA. 2002; 99:14682–14687. [PubMed: 12391305]
27. Nozaki M, Raisler BJ, Sakurai E, et al. Drusen complement components C3a and C5a promote choroidal neovascularization. Proc Natl Acad Sci USA. 2006; 103:2328–2333. [PubMed: 16452172]
28. Gupta N, Brown KE, Milam AH. Activated microglia in human retinitis pigmentosa, late-onset retinal degeneration, and age-related macular degeneration. Exp Eye Res. 2003; 76:463–471. [PubMed: 12634111]
29. Kimura H, Spee C, Sakamoto T, et al. Cellular response in subretinal neovascularization induced by bFGF-impregnated microspheres. Invest Ophthalmol Vis Sci. 1999; 40:524–528. [PubMed: 9950614]
30. Cui JZ, Chiu A, Maberley D, Ma P, Samad A, Matsubara JA. Stage specificity of novel growth factor expression during development of proliferative vitreoretinopathy. Eye. 2007; 21:200–208. [PubMed: 16531976]
31. Roque RS, Rosales AA, Jingjing L, Agarwal N, Al-Ubaidi MR. Retina derived microglial cells induce photoreceptor cell death in vitro. Brain Res. 1999; 836:110–119. [PubMed: 10415410]
32. Schwab C, Hosokawa M, McGeer PL. Transgenic mice overexpressing amyloid beta protein are an incomplete model of Alzheimer disease. Exp Neurol. 2004; 188:52–64. [PubMed: 15191802]
33. Zeng HZ, Zhu X, Zhang C, Yang LP, Wu L, Tso MOM. Identification of sequential events and factors associated with microglial activation, migration, and cytotoxicity in retinal degeneration in rd mice. Invest Ophthalmol Vis Sci. 2005; 46:2992–2999. [PubMed: 16043876]
34. Sierra A, Gottfried-Blackmore AC, McEwen BS, Bulloch K. Microglia derived from aging mice exhibit an altered inflammatory profile. Glia. 2007; 55:412–424. [PubMed: 17203473]

35. Wu KHC, Penfold PL, Billson FA. Effects of post-mortem delay and storage duration on the expression of GFAP in normal human adult retinae. Clin Exp Ophthalmol. 2002; 30:200–207. [PubMed: 12010215]

36. Duce JA, Hollander W, Jaffee R, Abraham CR. Activation of early components of complement targets myelin and oligodendrocytes in the aged rhesus monkey brain. Neurobiol Aging. 2006; 27:633–644. [PubMed: 15992964]

37. Gerl VB, Bobl J, Pitz S, Stoffelns B, Pfeiffer N, Bhakdi S. Extensive deposits of complement C3b and C5b-9 in the choriocapillaris of eyes of patients with diabetic retinopathy. Invest Ophthalmol Vis Sci. 2002; 43:1104–1108. [PubMed: 11923252]

38. Vogt SD, Barnum SR, Curcio CA, Read RW. Distribution of complement anaphylatoxin receptors and membrane-bound regulators in normal human retina. Exp Eye Res. 2006; 83:834–840. [PubMed: 16764856]

39. Penfold, PL., Wong, J., van Driel, D., Provis, JM., Madigan, MC. Immunology and age-related macular degeneration. In: Penfold, PL., Provis, JM., editors. Macular Degeneration. New York: Springer; 2005. p. 25-44.

40. Chen M, Forrester JV, Xu H. Synthesis of complement factor H by retinal pigment epithelial cells is down-regulated by oxidized photoreceptor outer segments. Exp Eye Res. 2007; 84:635–645. [PubMed: 17292886]

41. Fukumoto H, Tennis, Locascio JJ, Hyman BT, Growdon JH, Irizarry MC. Age but not diagnosis is the main predictor of plasma amyloid beta protein levels. Arch Neurol. 2003; 60:958–964. [PubMed: 12873852]

42. Bradt BM, Kolb WP, Cooper NR. Complement-dependent proinflammatory properties of the Alzheimer's disease-peptide. J Exp Med. 1998; 188:431–438. [PubMed: 9687521]

43. Donoso LA, Kim D, Frost A, Callahan A, Hageman G. The role of inflammation in the pathogenesis of age-related macular degeneration. Surv Ophthalmol. 2006; 51:137–152. [PubMed: 16500214]

44. Sivaprasad S, Chong NV. The complement system and age-related macular degeneration. Eye. 2006; 20:867–872. [PubMed: 16410816]

45. Funato H, Yoshimura K, Tamaoka A, et al. Quantitation of amyloid beta protein in the cortex during aging and in Alzheimer's disease. Am J Pathol. 1998; 152:1633–1639. [PubMed: 9626067]

46. Saido T, Iwata N. Metabolism of amyloid beta peptide and pathogenesis of Alzheimer's disease: towards presymptomatic diagnosis, prevention and therapy. Neurosci Res. 2006; 54:235–253. [PubMed: 16457902]

47. El Khoury JB, Moore KJ, Means TK, et al. CD36 mediates the innate host response to beta amyloid. J Exp Med. 2003; 197:1657–1666. [PubMed: 12796468]

48. Howes KA, Liu Y, Dunaief JL, et al. Receptor for advanced glycation end products and age-related macular degeneration. Invest Ophthalmol Vis Sci. 2004; 45:3713–3720. [PubMed: 15452081]

49. Tezel G, Luo C, Yang X. Accelerated aging in glaucoma: immunohistochemical assessment of advanced glycation end products in the human retina and optic nerve head. Invest Ophthalmol Vis Sci. 2007; 48:1201–1211. [PubMed: 17325164]

50. Herold K, Moser B, Chen Y, et al. Receptor for advanced glycation end products in a dash to the rescue. J Leukoc Biol. 2007; 82:204–212. [PubMed: 17513693]

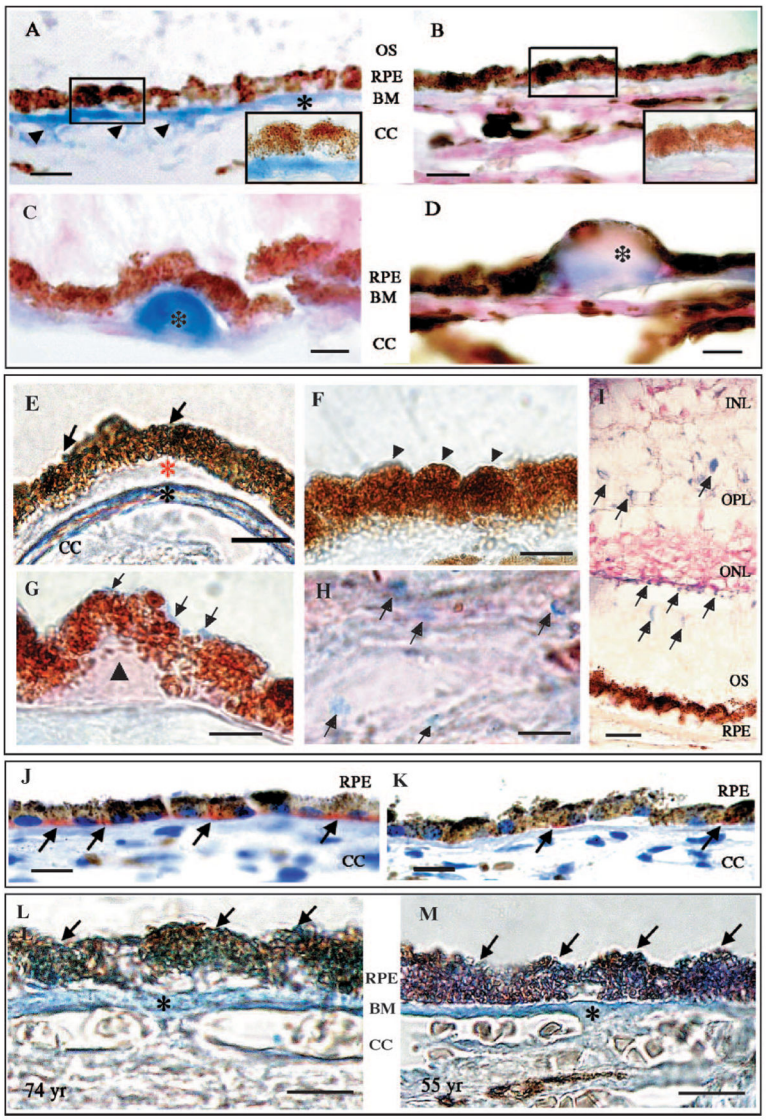

Figure 1.

(**A**–**D**) Immunoreactivity for C5b-9 in the RPE–choroidal complex. (**A**) Representative immunoreactivity of tissues from an eye in the older donor group. *Bright blue* (vector blue) reaction product demarcates positive immunoreactivity for the membrane attack complex, C5b-9. Note that Bruch membrane (*asterisk*) and the intercapillary pillars of Bruch (*arrowheads*) label intensely. The basal aspect of the RPE cell was faintly immunoreactive for C5b-9 (enlargement). (**B**) Background immunolabeling pattern is seen in tissues from eyes in the younger donor group. Note *faint blue* staining in Bruch membrane. (**C**, **D**) Occasional drusen sites (*asterisks*) were observed in the tissues from eyes of the older donor group. All drusen were strongly immunoreactive for C5b-9. Note that the RPE cells do not exhibit positive (*blue*) immunoreactivity even at higher magnifications, as shown here. Cryosections were counterstained with neutral red, resulting in a *light pink* stain of the choroid and retinal pigment epithelium. The RPE layer is *brown* because of endogenous pigment. (**E**–**H**) Immunoreactivity for CD11b in RPE–choroidal complex. Representative

cryosections from eyes of the older donor group are immunoreactive for the activated microglial marker, CD11b. Positive immunoreactivity is *bright blue* (vector blue) and is present on the apical surfaces of some RPE cells (**E**, **G**, *arrows*) and in Bruch membrane (**E**, *black asterisk*). Not all RPE cells were immunoreactive for CD 11b (**F**, *arrowheads*). Basal laminar deposit (**E**, *red asterisk*) and drusen (**G**, *black triangle*) remain negative for CD11b. Several perivascular cells are CD11b immunoreactive in the choroid (**H**, *arrows*). (**I**) RCA-I binding in neural retina. Note vector blue–positive profiles scattered throughout the outer plexiform layer (OPL), outer segment layer (OS), and at the border between the outer nuclear layer (ONL) and OS. (**J**–**K**) Immunoreactivity for APP in RPE–choroidal complex in paraffin-embedded sections. (**J**) Representative section from an eye of the older donor group demonstrating strong immunoreactivity for APP. *Arrows* point to the *intense red* reaction product (AEC) in the basal cytoplasmic compartment of the RPE cell. (**K**) Representative section from an eye of the younger donor group reveals a weaker immunoreactive labeling pattern. Two RPE cells (*arrows*) demonstrate APP immunoreactivity confined to the basal cytoplasm. Sections were counterstained with hematoxylin (*blue*). (**L**, **M**) Immunoreactivity for the scavenger receptor, CD36, in the RPE–choroidal complex. Immunoreactivity for CD36 did not demonstrate age dependence. Several examples indicated strong immunoreactivity for CD36 in older (**L**) and in younger (**M**) donor eyes, as shown by the *bright blue* reaction product (vector blue). CD36 was strongly immunoreactive on the retinal pigment epithelium (*arrows*), Bruch membrane (BM), and intercapillary pillars of Bruch (*asterisk*) and moderately immunoreactive in the choriocapillaris (CC). Scale bar: (**A**, **B**, **I**–**K**) 20 μm; (**C**, **D**, **E**–**H**, **L**, **M**) 10 μm.

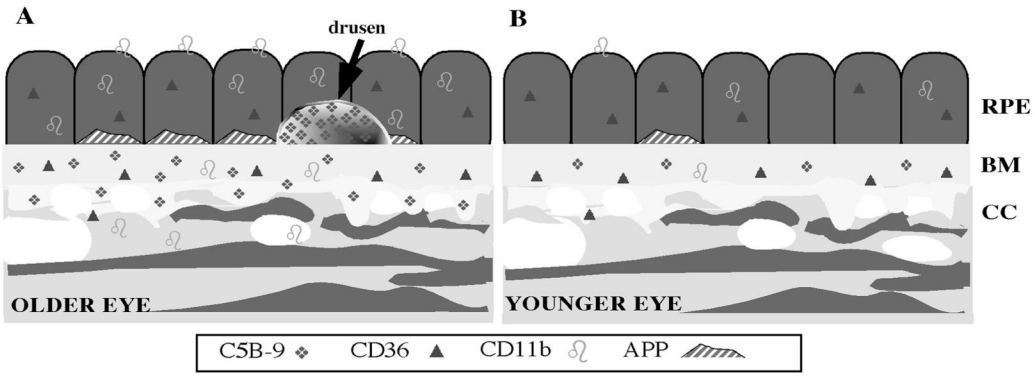

Figure 2.

Summary diagram depicting the distribution of C5b-9, CD36, CD11b, and APP immunoreactivity in the RPE–choroidal complex of older and younger eyes. Older eyes demonstrated significantly stronger immunoreactivity for C5b-9 in Bruch membrane and the intercapillary pillars of Bruch. Drusen, found in the older eyes in this study, were also immunoreactive for C5b-9. RPE cells demonstrating APP immunoreactivity were more numerous in older eyes. Activated microglia immunoreactivity was significantly greater in the RPE layer, the connective matrix of the choroid, and Bruch membrane of the older eyes. CD36 immunoreactivity was present in eye tissues of both age groups.

Table 1

Proteins Studied and Antibodies Used

Protein/Marker of Inflammation	Description	Primary Antibody or Lectin	Specificity	Manufacturer
C5b-9/membrane attack complex (MAC)	The terminal product of the complement cascade; signals complement activation	Monoclonal mouse anti-human C5b-9 clone aE11	Antibody labels poly-C9 and, to a lesser degree, C9 but does not recognize C5, C6, C7, or C8 components	Dako, Glostrup, Denmark
CD36	Scavenger receptor expressed on RPE cells; aids in cellular homeostasis and clearance of aberrant lipoproteins; confers protection against pathogens and inflammation	Monoclonal mouse anti-human CD36	Antibody recognizes membrane glycoprotein GPIIIb found on monocytes/ macrophages and platelets	Chemicon, Temecula, California
CD11b	Transmembrane protein that promotes phagocytosis of cells coated with iC3b or IgG; CD11b immunoreactivity targets activated microglial populations	Monoclonal mouse anti-human CD11b	Antibody recognizes CD11b antigen, which associates with CD18 to form Mac-1	Invitrogen, Carlsbad, California
Amyloid precursor protein (APP)	Implicated in the formation of amyloid beta, a candidate trigger of complement activation; expressed in many cell types, including the RPE cell	Monoclonal mouse anti-APP A4 antibody clone 22C11	Antibody reacts with the pre-A4 molecule, which is a component of extracellular amyloid and is derived from the larger peptide APP; recognizes all 3 isoforms of APP (immature, sAPP, mature)	Chemicon, Temecula, California
Ricinus communis agglutinin-I (RCA-I)	Plant lectin that selectively binds to oligosaccharides ending in D-galactose or N-acetylgalactosamine; used to mark quiescent and activated microglia cells in the retina	Biotinylated RCA-I lectin	Lectin reacts with the cell bodies and microglial processes	Vector Laboratories, Burlingame, California

Table 2

Semiquantitative Analysis of C5b-9, CD36, CD11b, and APP Immunoreactivity and RCA-I Binding

Donors	Age (years)	Sex	C5b-9 Frozen	RCA-I Frozen	CD36 Frozen	CD11b Paraffin	APP Paraffin	Cause of Death
Younger								
1	7	M	−	−	++	NA	NA	Head trauma
2	43	M	−	−	−	NA	NA	Lung cancer
3	46	M	−	−	−	NA	NA	Lung cancer
4	46	M	−	−	++	NA	NA	Cardiomyopathy
5	48	M	++	−	+	NA	NA	Cardiomyopathy
6	48	F	−	−		NA	NA	Lung cancer
7	48	F	−	−	++	NA	NA	Myocardial infarction
8	48	F	−	++		NA	NA	Breast cancer
9	50	M	−	++	+	NA	NA	Germ cell cancer
10	52	F	++	−	++	NA	NA	Cervical cancer
11	52	F	−	−	++	NA	NA	Colon cancer
12	53	F	−			NA	NA	Lung cancer
13	53	M	−	−	+	NA	NA	Colon cancer
14	54	F	−	−	+++	NA	NA	Lung cancer
15	55	M	−	++	+++	NA	NA	Laryngeal cancer
16	46	F	NA	NA	NA	++	+	Lung cancer
17	47	F	NA	NA	NA	+	−	Lung cancer
18	48	F	NA	NA	NA	+	−	Liver cancer
	56	F	NA	NA	NA	−	NA	Breast cancer
Older								
1	69	M	NA	++	NA	NA	NA	Liver cancer
2	70	F	+++	++	NA	NA	NA	Myocardial infarction
3	70	F	++	++	NA	NA	NA	Diabetes
4	70	M	−	++	++	NA	NA	Gastric cancer
5	70	M		+	++	NA	NA	Rectal cancer
6	70	F	−	NA	+++	NA	NA	Breast cancer
7	70	M	NA	NA	++	NA	NA	NA

Donors	Age (years)	Sex	C5b-9 Frozen	RCA-I Frozen	CD36 Frozen	CD11b Paraffin	APP Paraffin	Cause of Death
8	71	F	++	NA	NA	NA	NA	Congestive heart failure
9	71	M	NA	NA	++	NA	NA	NA
10	71	M	+	+	+++	NA	NA	NA
11	71	F	−	NA	NA	NA	NA	Lung cancer
12	72	M	−	+++	+	NA	NA	Prostate cancer
13	72	M	+	+++	NA	NA	NA	Congestive heart failure
14	72	M	++	NA	NA	NA	NA	NA
15	72	F		NA	+	NA	NA	NA
16	73	M		NA	++	NA	NA	NA
17	73	M	+++	+++	++	NA	NA	Laryngeal cancer
18	74	M	+++	++	NA	NA	NA	Esophageal cancer
19	74	M	++	++	+++	NA	NA	Prostate cancer
20	74	M	+	−	++	NA	NA	Lung cancer
21	74	M	++		+	NA	NA	Renal failure
22	74	F	NA	NA	++	NA	NA	
23	70	M	NA	NA	NA	+	+	Prostate cancer
24	72	M	NA	NA	NA	++	+	Cerebral vascular insult
25	72	M	NA	NA	NA	+++	+++	Lung cancer
26	73	M	NA	NA	NA	++	+++	Prostate cancer
27	73	M	NA	NA	NA	−	NA	Lung cancer
28	73	M	NA	NA	NA	++	NA	Pulmonary fibrosis
29	73	M	NA	NA	NA	+++	NA	Lung cancer
30	74	M	NA	NA	NA	++	NA	Bowel cancer
31	80	M	NA	NA	NA	+++	+++	NA

t-Test (one-tail unequal variance) values were as follows: C5b-9, $P = 0.0009$; RCA-1, $P = 0.0001$; CD36, $P = 0.1178$; CD11b, $P = 0.0004$; APP, $P = 0.0227$. NA, not available.