Increased choroidal mast cells and their degranulation in age-related macular degeneration

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Abstract

Background/Aims—Inflammation has been implicated in age-related macular degeneration (AMD). This study investigates the association of mast cells (MCs), a resident choroidal inflammatory cell, with pathological changes in AMD.

Methods—Human donor eyes included aged controls (n=10), clinically diagnosed with early AMD (n=8), geographic atrophy (GA, n=4), and exudative AMD (n=11). The choroids were excised and incubated alkaline phosphatase (APase; blood vessels) and nonspecific esterase activities (MCs). Degranulated (DG) and nondegranulated (NDG) MCs in four areas of posterior choroid (nasal, nonmacular, paramacular, and submacular) were counted in flat mounts (4∼6 fields/area). Choroids were subsequently embedded in JB-4 and sectioned for histological analyses.

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Results—The number of MCs was significantly increased in all choroidal areas in early AMD (p=0.0006) and in paramacular area in exudative AMD (139.44±55.3 cells/mm^2; p=0.0091) and GA (199.08±82.0 cells/mm^2; p=0.0019) compared to the aged controls. DG MCs was also increased in paramacular (p=0.001) and submacula choroid (p=0.02) in all forms of AMD. Areas with the greatest numbers of DG MC had loss of choriocapillaris (CC). Sections revealed that the MCs were widely distributed in Sattler's and Haller's layer in the choroidal stroma in aged controls, whereas MCs were frequently found in close proximity to CC in GA and exudative AMD and in choroidal neovascularization (CNV).

Conclusion—Increased MC numbers and degranulation were observed in all AMD choroids. These results suggest that MC degranulation may contribute to the pathogenesis of AMD: death of CC and RPE and CNV formation. The proteolytic enzymes released from MC granules may result in thinning of AMD choroid.

Keywords

Age-related macular degeneration; choriocapillaris; choroid; degranulation; mast cells

Introduction

Age-related macular degeneration (AMD) is a complex, multifactorial disease characterized by progressive degeneration of the photoreceptor/retinal pigment epithelium/choriocapillaris complex primarily in the macular region of the eye, resulting in irreversible central vision loss among the elderly population.[1] Although the etiology for AMD remains unclear, accumulating evidence suggests that inflammation plays an important role in its pathogenesis. Histopathological studies of human choroids confirm the presence of inflammatory changes, but reveal little regarding the origins of the inflammation. Polymorphisms in complement factor H and other complement factors have been found to be a risk factor in AMD, adding to the evidence that inflammation is involved in AMD.[2]

Mast cells (MCs) are key effector cells of inflammation and play an important role in innate and acquired immunity, as well as autoimmunity.[3] These cells are derived from multilineage hematopoietic progenitors that migrate to vascularized tissues and organs, where they mature and ultimately reside.[4] Mature MCs have high-affinity IgE receptors (FcεRI) on their cell membrane surface, and the cross-linking of these receptors appears to act as a trigger for MC degranulation in IgE-mediated hypersensitivity reactions. Complement 3a and 5a have anaphylatoxin activity and contribute to degranulation of MCs.[5] Activation triggers degranulation of MCs, releasing histamine, cytokines, chemokines, and proteases into the milieu.[6 7] The proteases include chymase and tryptase that are responsible for the immediate hypersensitivity response to allergens and they activate matrix metalloproteinases, which can degrade stroma and basement membranes.

MCs are widely distributed in connective tissue and mucosa and are frequently found in close proximity to blood vessels. MCs are abundant in the human choroid where they are the connective tissue type MC.[8 9] Since MCs play a key role in inflammation in other tissues like skin and conjunctiva, MCs may play a role in choroidal inflammation as well.[10] MCs can interact with endothelial cells (ECs) and induce their proliferation through release of

heparin, metalloproteinases, and VEGF.[11-13] MCs have the ability to induce angiogenesis and have been demonstrated to be present around Bruch's membrane during both the early and late stages of choroidal neovascularization (CNV) in AMD.[14] Interestingly, oral tranilast, an antiallergic drug that inhibits the release of chemical mediators from MCs has been shown to suppress laser-induced CNV in the rat.[15] In order to understand the role of MCs in AMD pathology, the total number of choroidal MCs [degranulated (DG) and nondegranulated (NDG)] was determined in aged control and AMD eyes. This study compared MC populations in submacular, paramacular, nonmacular, and nasal choroid and the relationship of MCs to the AMD-associated pathology.

Subjects and Methods

Donor eyes

All procedures in this study adhered to the tenets of the Declaration of Helsinki regarding research involving human tissue and were approved by the institutional review boards of the Johns Hopkins School of Medicine and Tufts Medical Center. Human donor eyes were obtained from the ongoing genetic and epidemiologic studies of Dr. Johanna Seddon (n=19 from New England Eye Center, Tufts Medical Center, Boston, MA, USA) and patient population of Dr. Janet Sunness (Greater Baltimore Medical Center, Baltimore, MD, USA) and from the National Disease Research Interchange (NDRI; Philadelphia, PA, USA) within 6–30 hours of death. Choroids from 23 subjects clinically diagnosed with AMD (age range 73–102 years; mean age 84.8±10.1 years) and from 10 aged control donors (age range 53–93 years; mean age 77.7±13.3 years) with no evidence of macular disease were studied. All donors were Caucasian. Table 1 summarizes the characteristics of each subject. The severity and phenotype of AMD was categorized according to our previously reported grading system,[16] using clinical records and ocular images, by reviewing ocular medical history on the eye bank transmittal sheets and by the post mortem gross examination of posterior eyecup, using transmitted and reflected illumination with a dissecting microscope (Stemi 2000; Carl Zeiss Meditec, Inc., Thornwood, New York, USA). During gross examination, AMD eyes were classified as follows: no AMD (Grade 1, without drusen or with fewer than 10 small drusen and without pigment abnormalities)(n=10); early AMD (Grades 2 and 3 combined for the purpose of these analyses, grade 2 had several small drusen with or without pigmentary change and grade 3 had large drusen)(n=8); dry or non-exudative AMD with geographical atrophy (GA, Grade 4, atrophy 350 μm characterized by distinct areas of retinal pigment epithelial (RPE) loss (n=4); or wet (exudative) AMD (Grade 5, with evidence of RPE detachment and/or CNV)(n=11).[16]

Tissue preparation

The globes were opened at the limbus and then the anterior segment of the eye was removed. The posterior eyecup was grossly examined and digital images were captured as previously published.[17] After removal of vitreous, the choroid with sclera was placed in 1% ethylenediaminetetraacetic acid (EDTA; J. T. Baker Chemical Co., Center Valley, PA, USA) in Ca^{2++}/Mg^{2++} free phosphate-buffered saline, pH 7.4, at room temperature for 2 h to remove RPE. Gross digital images of choroid were again captured with and then without RPE. The choroid was then dissected from the sclera at the lamina fusca and fixed in 2%

paraformaldehyde in 0.1 M cacodylate at 4°C for 1 h, washed in several changes of 0.1 M cacodylate buffer at 4°C, and then incubated for the alkaline phosphatase (APase) activity as previously described.[18] After APase staining, nonspecific esterase enzyme (NSE) staining was performed using a naphthol AS-D chloroacetate kit (Sigma Diagnostics, St. Louis, MO, USA).[17] This method preferentially stains granulocytes and MCs bright red. The two cell types can be easily distinguished since MCs are 4 times larger than granulocytes. The APase and NSE stained choroid was post-fixed in 2% paraformaldehyde for 24 h, then washed and melanin was bleached with 30% hydrogen peroxide (Sigma-Aldrich Co.) at 4°C as previously reported.[18]

Mast Cell Counts

A total of 23 AMD and 10 aged control choroids were counted for the number of MCs. After flat-mounting the wet choroid, the posterior pole choroid (7mm × 5mm) was excised and analyzed for MC numbers. The tissue included was from 1 disc diameter (DD) nasal to 6 DD temporal to the disc and 2.5 DD inferior and superior to the disc (supplemental figure 1). Within this piece of choroid, four areas were evaluated (nasal, nonmacular, paramacular, and submacular). Using a Zeiss Photomic 2 microscope at 16× magnification (field area = 1 mm^2), total number of MCs (DG and NDG) in each choroid was counted manually in four to six random microscopic fields and then expressed as number/mm^2 in each area. More than 20 fields were counted and documented per choroid. Areas of viable capillaries were determined by the presence of APase$^+$ microvasculature. MCs were stained intensely red after NSE incubation and they appeared rounded in choroid stroma under brightfield illumination. MC numbers were compared in pathological and non-pathological areas of choriocapillaris (CC). Pathological areas were defined as areas that had CC dropout (loss in APase activity) and/or apparent CNV (irregular vascular patterns with abnormally high APase activity). The density of the choroidal vasculature was determined by image analysis of images from the APase/NSE flat mounts as previously published.[19]

Flat-embedding of Choroid

After flat perspective analysis, two choroids from each group were post-fixed flat in 25% Karnovsky's glutaraldehyde/paraformaldehyde, washed, dehydrated as described previously, [18] and infiltrated and embedded in freshly catalyzed glycol methacrylate (JB-4; Polysciences, Warrington, PA, USA), as recommended by the manufacturer. Sections (2.5 μm) were cut with a dry glass knife on a Sorvall NTB2 microtome. Morphometric analysis was done with a Zeiss Photomic II microscope. Choroidal thickness (Bruchs membrane to lamina fusca) was measured in 5 fields of each image and 5 measurements/field in submacular choroid in sections.

Statistical analysis

The number of MCs/mm^2 for all fields in each area of each group of subjects was averaged and expressed as the means ± SEM/mm^2 of choroid. The p values were determined by comparing mean numbers from the aged control choroids with numbers from choroids with either early AMD, CNV or GA using the Welch's t-test, which assumes unequal variance and the Wilcoxon rank sum (WRS) test. A p value <0.05 was considered statistically significant.

Results

Double labeling with APase and NSE resulted in choroids with blue-stained blood vessels and bright red-stained MCs (figure 1B-C). Neutrophils were also stained red but were 3-4 times smaller than mast cells and mostly confined to blood vessel lumens. NDG MCs were large round or oval, bright red cells. We have previously determined that loss in APase activity represents loss of viable endothelial cells and, therefore, CC dropout.[18] No or very small areas of CC dropout were observed in choroids from aged control subjects (figure 1A). MCs were not present at the level of CC but were rather obvious around blood vessels in Haller's and Sattler's layer deeper in the flat choroid (figure 1B-D). In aged control choroids, the total number of MCs (DG and NDG; mean±SEM) in nasal, nonmacular, paramacular, and submacular areas was 90.97 ± 48.8 cells/mm^2, 97.69 ± 42.3 cells/mm^2, 80.08 ± 34.8 cells/mm^2 and 64.87 ± 37.04 cells/mm^2 respectively (figure 1E-H). Most MCs were not degranulated in aged control choroids. In aged control choroids, histologic sections confirmed that the MCs were widely distributed in Sattler's and Haller's layers in the choroidal stroma but rarely at the level of CC (figure 1D).

In choroids from early AMD, some CC channels were not viable (APase-) in the submacular area. In Subject #15 who was graded 2C, having drusen and pigmentary changes (figure 2A), the area of CC loss was predominantly nasal to the optic nerve and submacula. In areas of capillary loss in early AMD, we have often observed apparent buds of intrachoroidal neovascularization (figure 2B). Many MCs in early AMD were degranulated having a larger irregular shape and free NSE$^+$ granules surrounding the cell soma (figure 2C). The total number of MCs was significantly increased in all areas in early AMD compared to controls (p values from p=0.032 to p=0.0006) and DG MCs were also increased significantly except in nonmacular areas (figure 1E-H). Degranulation was also apparent in the sections from early AMD choroids and MCs appeared to be located more anteriorly, near CC or in open spaces created after CC degeneration (figure 2D-F).

High levels of APase blue reaction product were observed in the endothelial cells of viable CNV (figure 3A) and CC loss was observed at the borders of CNV in exudative AMD (figure 3A-B). Large numbers of MCs were degranulated in the exudative AMD choroids (115.1 ± 61.3; p=0.0032 [WSR]) and the numbers of DG MCs were significantly increased in all areas compared to control subjects (p values from p=0.0032 to 0.024)(figure 1E-H). In sections, MCs were observed adjacent to CNV feeder vessels where they traversed breaks in Bruchs membrane (figure 3D-E) and in the basal laminar deposit overlying CNV or even within the CNV itself (figure 3F-G).

The most severe areas of CC dropout were noted in GA subjects in regions of RPE atrophy (figure 4A-B). The largest total numbers of MCs overall in the study were counted in GA paramacular regions (199.08 ± 82.0 cells/mm^2; p=0.0019) and nasal ($192.84+108.6$ cells/mm^2; p=0.028) areas (figures 1E-H and 4). In addition all areas of GA choroids had significantly elevated numbers of DG MCs compared to controls (p values from p=0.0073-0.012). In border regions and areas lacking RPE atrophy, MCs were at the level of Sattler's layer (figure 4F) but also at the level of CC in regions with complete RPE atrophy (figure 4D-E).

In summary, degranulation of MCs was increased in submacular (WRS test p=0.02) and in paramacular choroid (WSR test, p=0.001) and in all forms of AMD (figure 1E-H). Areas with the greatest numbers of DG MC had a significant reduction in vascular area, i.e. had loss of CC (data not shown). Also, in AMD choroids, DG MCs were frequently found in close proximity to CC and adjacent to degenerated CC (lacked APase activity)(figures 2D-F and 3D-E) and were present in abundance in exudative AMD choroid (figure 4).

Discussion

A central role for inflammation in AMD has been proposed. This is based on the findings that there are many genes in the alternative complement pathway that are risk factors for AMD.[20] The resident inflammatory cells of choroid are tissue macrophages and mast cells.[9] This is the first report that mast cell numbers and activation are increased in AMD. This increase occurred in all forms of AMD, including early AMD. Furthermore, the numbers presented are probably underestimations of the increase because the numbers were counted in flat mounts by area of choroid (mm^2) but all forms of AMD have thinning of choroid [21]. Therefore, counting MCs per volume of choroid, i.e. mm^3, would likely result in more significant increases in numbers in AMD.

MC activation results in degranulation, the release of a myriad of factors and enzymes into the milieu. MCs are chemotactic to and activated by C3a and C5a,[5] by- products of complement activation, which are present in drusen in AMD.[22] MCs can be also be activated by a wide range of stimuli, including immunoglobulins via Fc receptors (FcεRI, FcγR), microbial pattern-recognition receptors (PRRs, such as TLRs), neuropeptides, cytokines, growth factors, toxins, venoms or venom components and physical stimuli.[23] These stimuli trigger MCs to release a diverse array of biologically active products via granular release and simple secretion, many of which can potentially mediate pro-inflammatory, anti-inflammatory and/or immunosuppressive functions.[24] This would contribute to the already proinflammatory choroidal environment in aging and AMD, downregulation of CFH and upregulation of CRP,[25] which can also activate MCs.[26] MCs can participate in multiple cycles of activation for mediator release and can be differentially activated to release distinct patterns of mediators or cytokines, depending on the type and strength of the activating stimuli.[24]

The consequences of activation and degranulation are myriad. Smith, as early as 1966, found that inducing choroidal MC degranulation resulted in increased blood flow probably as a result of histamine release.[27] More recently, Bousquet and associates observed ocular inflammation and increased vascular dilation and permeability in choroidal vessels after inducing degranulation.[10] This is accomplished by the release of chemokines and cytokines that attract and activate macrophages and T-cells [23], which is well documented in cardiovascular disease, atherosclerosis, and aortic aneurysms.[5]

MCs populate the connective tissue near blood vessels in many tissues, positioned as key elements in processes like wound healing, tissue regeneration, and remodeling after injury, fibrosis, and angiogenesis. These observations suggest an interaction between MCs and ECs; indeed, many MC-derived mediators are angiogenic. MCs in the human intima and

adventitia express basic fibroblast growth factor and produce angiogenic histamine, leptin, VEGF, and angiopoietin-1.[23] MC tryptase stimulates microvessel tube formation and enhances the growth of microvessel EC, whereas chymase promotes angiogenesis through its effects of angiopoietin-II.[28 29] In this study, MCs appeared within and adjacent to CNV in exudative AMD choroids (figure 4). Tryptase and chymase activate matrix metalloproteinases (MMPs), which degrade stroma and basement membranes,[30] and in doing so may be responsible for the death of smooth muscle and endothelial cells and RPE, cells dependent on an intact basement membrane.[5] Sohn and associates have observed disruption of the stromal ultrastructure in thin choroids of AMD subjects, suggesting degradation of choroidal stroma in AMD.[31] Therefore, MMP activation and increased proteolysis after degranulation may cause choroidal thinning and loss of CC and RPE, all of which occur in GA. Godfrey hypothesized that choroidal mast cells may also be involved in fibrosis and scarring, as they are in skin, and as occurs in end stage exudative AMD.[32]

In conclusion, MCs contain an abundance of vasoactive, proinflammatory, anticoagulant, and proteolytic mediators that are released upon activation and degranulation, which was increased in all AMD groups.[23] The proteolytic enzymes released by MCs may lead to thinning of the choroid in AMD as well as degradation of vascular basement membranes and Bruch's membrane, which in turn could result in RPE death and choriocapillaris degeneration that we have documented in GA and exudative AMD.[1 19] In addition, MC degranulation releases angiogenic factors that may contribute to formation of CNV. Therefore, the increase in MCs and MC degranulation observed and the release of various soluble and granular factors may contribute to the pathogenesis of AMD, suggesting that MC stabilization may be a therapeutic goal in controlling progression of AMD.

Acknowledgments

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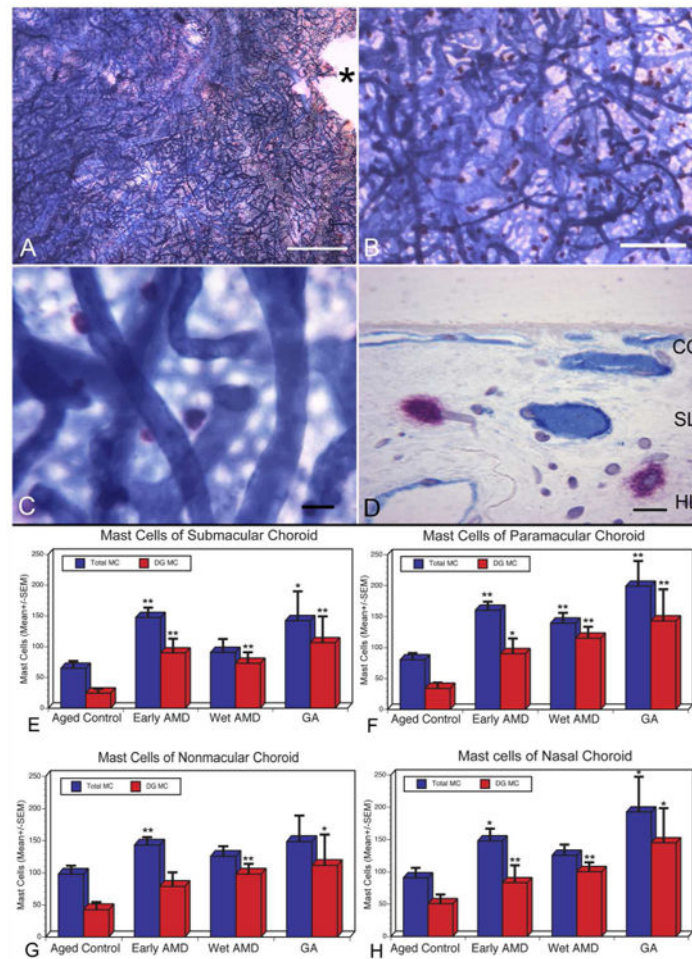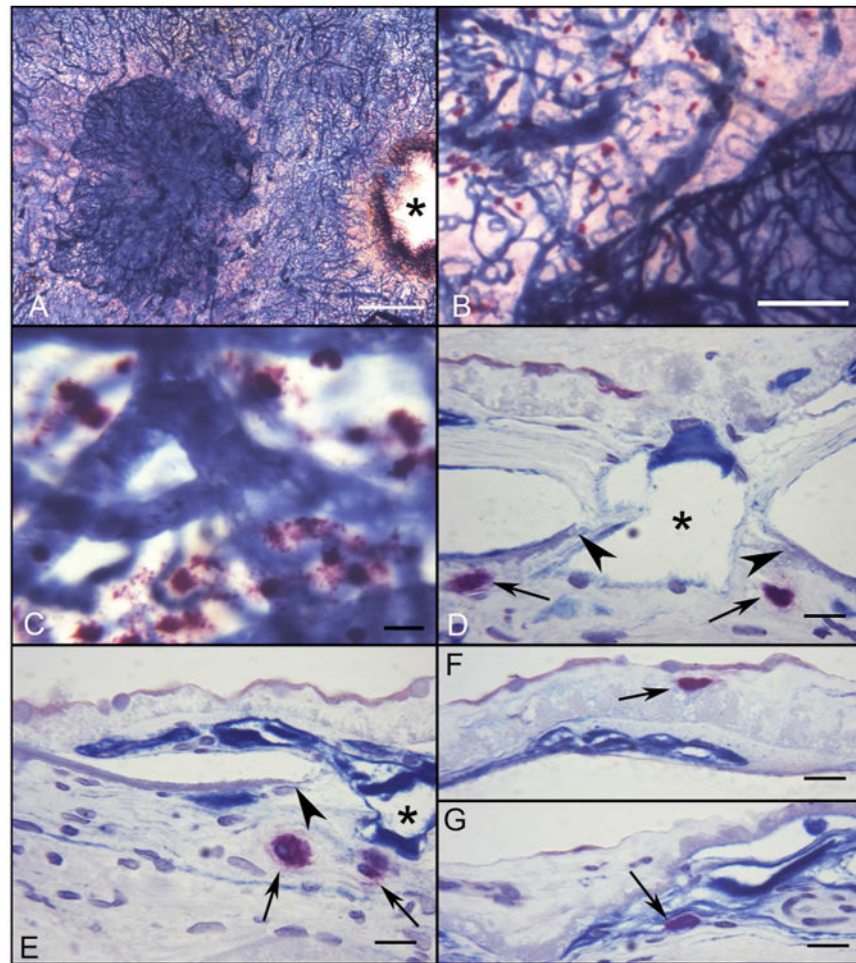
Figure 1.
Flat mount of submacular choroid from an aged control subject stained with alkaline phosphatase and nonspecific esterase (APase/NSE) (Subject#4). (A and B) APase$^+$ choroidal vessels are stained blue and NSE-positive MCs are stained red with this preparation. (C) MCs are largely distributed in the intermediate and deep choroid so the choriocapillaris (CC) is out of focus. (D) Histological section of the choroid shown in (A) demonstrates red MCs are associated with blood vessels in Sattler's layer (SL) and Haller's layer (HL), which are APase$^+$ and therefore have viable endothelial cells.(*, optic nerve; Bar=1mm in A, 200μm in B, 50μm in C, and 20mm in D). (E-H) Comparison of the number of MCs (total MCs, blue bar; degranulated or DG MCs, red bar) present per mm^2 of choroid counted in the flat perspective in aged control and AMD subjects. MC numbers/mm^2 are presented from four areas of posterior choroid [submacular (E), paramacular (F), nonmacular (G), and nasal (H)] and compared to the aged control in those regions. Total numbers of MCs as well as degranulated MCs were statistically significantly increased in almost all areas examined in AMD choroids compared to the aged control. The significance of the difference between aged control and AMD (p< 0.05) is indicated using the Students t-test (*) and Wilcoxon rank-sum test (**).
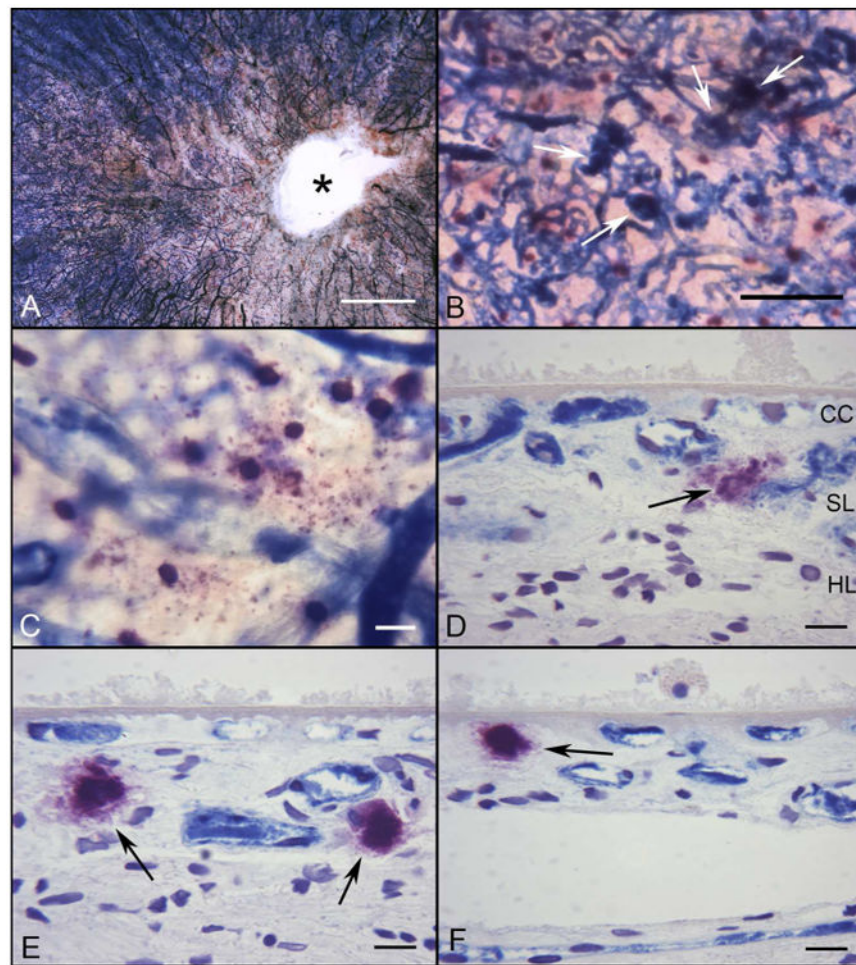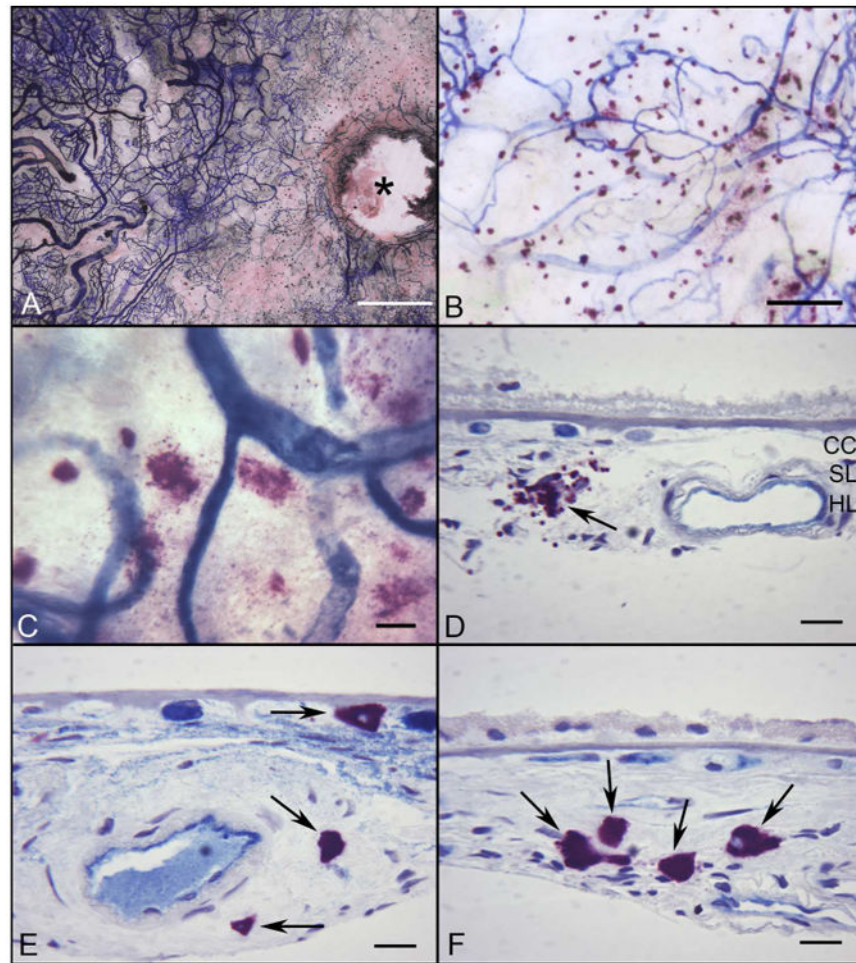
Figure 2.
Flat mount of APase/NSE stained choroid from a subject with early AMD (Subject#15). A succinct area of CC degeneration (lacking APase staining) is evident in the submacular region and around the optic disc(*)(A). Several neovascular buds have dark blue APase activity (B, white arrows). (C) At higher magnification, some degranulated MCs are apparent at the level of CC, which has reduced APase activity in this area. (D, E, F) Histological sections from same choroid demonstrate the DG MCs (black arrows) present near degenerative CC channels, which are APase⁻ and around the dark blue APase⁺ neovascular buds. A thin basal laminar deposit is present internal to Bruchs membrane. (Bar=1mm in A, 200μm in B, 50μm in C, and 20μm in D-F)

Figure 3.
Flat mount of APase/NSE choroid from a subject with wet AMD (Subject#20). A large APase stained choroidal neovascular formation (CNV) is present in submacular region of the choroid (A)(*, optic nerve). (B) Numerous degranulated MCs are adjacent to the edge of CNV, where the CC is greatly attenuated. (C) At higher magnification, degranulated MCs are present at the edge of CNV. (D-G) Histological sections from same choroid demonstrate the degranulated MCs (black arrows) adjacent to the CNV feeder vessel. An MC is present in a basal laminar deposit (F) and in the CNV itself (G). Breaks in Bruch's membrane are indicated by arrowheads. (*, CNV breaching Bruchs membrane; Bar=1mm in A, 200μm in B, 50μm in C, and 20μm in D-G)

Figure 4.
Flat mount preparation of APase/NSE incubated choroid from a subject with GA
(Subject#30) (A) There is a large area of CC loss (APase⁻) around the optic disc (*) and in
the submacular region of the choroid. (B) Many degranulated MCs are associated with areas
of RPE atrophy and CC degeneration. (C) At higher magnification, most MCs are
degranulated. (D-F) Histological sections from same choroid demonstrate the degranulated
MCs (black arrows) aggregating in choroidal stroma in a region of RPE atrophy with a thin
basal laminar deposit (D) and in the wall of an artery with atherosclerotic changes (E). MCs
are increased in number but in their normal position in Sattler's and Haller's layers in a
region without RPE atrophy (F). (Bar=1mm in A, 200μm in B, 50μm in C, and 20μm in D-
F)

Table 1

Characteristics of human subjects

Subjects	Time (hours)		Age/race/sex	Primary cause of death	Medical history
	DET	PMT			
Aged Control					
1	3.5	25	53/C/F	Breast cancer	Mets to brain & liver, pleural effusion, Crohn's disease
2	4.2	31	78/C/M	Prostate cancer	COPD, HTN, CAD, MI, emphysema
3	3	29	93/C/F	COPD	CAD, CHF, COPD, HTN
4	6.5	28	77/C/M	Respiratory failure	Alzheimer's disease, dementia, CAD, DM, HTN
5	3.8	26	59/C/M	Respiratory failure	Pneumonia, MI, schizophrenia, anemia
6	5	29	88/C/F	CHF	CVA, CHF, MI, renal insufficiency
7	4	24	84/C/M	Pancreatic cancer	AFIB, CVA, CHF, CAD, PVD
8	4.5	32	80/C/F	Coronary artery disease	Dementia, CVD, gastroesophageal reflux
9	3.3	23	86/C/M	MI	Osteoarthritis, CVA, arthritis
10	4	24	79/C/F	Unknown	COPD, dementia
Early AMD					
11	6.5	25	79/C/M	COPD	Mild NIDDM, CHF, CVA, CAD, COPD, arthritis
12	5	31	73/C/F	Respiratory failure	Anemia, arthritis, HTN, gastroesophageal reflux
13	4	36	81/C/M	CAD	AFIB, CAD, peptic ulcer
14	16	35	86/C/M	COPD & enlarged heart	COPD, smoker
15	8	31	92/C/M	CHF	CHF, PVD, arthritis, eczema, renal disease, skin cancer
16	18	37	91/C/M	Heart failure	PVD, arthritis
17	10	48	86/C/F	Dementia/Alzheimer's	Arthritis, colitis
18	10	50	94/C/M	Heart attack	HTN, DM, arthritis, prostate cancer
Late AMD (Wet)					
19	6	26	91/C/F	Heart failure	HTN
20	8	24	91/C/M	Dementia, heart failure	Heart attack, colitis
21	8	28	90/C/F	CHF, kidney transplant complications	DM, HTN, CAD, PVD
22	13	35	100/C/F	Heart failure	HTN, stroke, arthritis

Subjects	Time (hours)		Age/race/sex	Primary cause of death	Medical history
	DET	PMT			
23	6	23	97/C/F	Alzheimer's disease	DM, HTN, stroke, arthritis,
24	4.5	24	97/C/F	Lung cancer	osteoporosis, eczema
25	5.5	24	102/C/F	CHF	HTN, CHF, reflux
26	2	23	86/C/M	Acute respiratory failure	HTN, skin cancer, angina
27	28	48	96/C/F	Respiratory	AFIB, DM, Parkinson's,
28	14	35	96/C/F	failure/pneumonia	esophageal reflux
29	5.3	17	85/C/M	Unknown Esophageal cancer	Breast cancer DM, HTN, arthritis, heart attack, osteoporosis HTN, stroke, shingles
Late AMD (GA)					
30	6	9	88/C/M	CHF	CAD, HTN, MI, GI bleed
31	3	24	81/C/F	Liver disease	Asthma, DM, HTN, arthritis, COPD, osteoporosis, liver cancer
32	17	40	87/C/M	CHF	HTN, DM, myocardial infarction,
33	12	38	97/C/F	Spinal stenosis & CHF	arthritis, prostate cancer CHF, PVD, arthritis, HTN, stroke, osteoporosis, angina

DET, death to enucleation time; PMT, postmortem time (death to fixation); C, Caucasian; M, male; F, female; AMD, age-related macular degeneration; CVA, cerebrovascular accident; DM, diabetes mellitus; NIDDM, non-insulin dependent diabetes mellitus; HTN, hypertension; COPD, chronic obstructive pulmonary disorder; GA, geographic atrophy; MI, myocardial infarction; CAD, coronary artery disease; CHF, congestive heart failure; AFIB, arterial fibrillation; PVD, Pulmonary vascular disease; GI, gastro-intestinal.