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Retinal pigment epithelial expression of complement regulator CD46 is altered early in the course of geographic atrophy

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Abstract

In geographic atrophy (GA), the non-neovascular end stage of age-related macular degeneration (AMD), the macular retinal pigment epithelium (RPE) progressively degenerates. Membrane cofactor protein (MCP, CD46) is the only membrane-bound regulator of complement expressed on the human RPE basolateral surface. Based on evidence of the role of complement in AMD, we hypothesized that altered CD46 expression on the RPE would be associated with GA development and/or progression. Here we report the timeline of CD46 protein expression changes across the GA transition zone, relative to control eyes, and relative to events in other chorioretinal layers. Eleven donor eyes (mean age 87.0 ± 4.1 yr) with GA and 5 control eyes (mean age 84.0 ± 8.9 yr) without GA were evaluated. Macular cryosections were stained with PASH for basal deposits, von Kossa for calcium, and for CD46 immunoreactivity. Internal controls for protein expression were provided by an independent basolateral protein, monocarboxylate transporter 3 (MCT3) and an apical protein, ezrin. Within zones defined by 8 different semi-quantitative grades of RPE morphology, we determined the location and intensity of immunoreactivity, outer segment length, and Bruch's membrane calcification. Differences between GA and control eyes and between milder and more severe RPE stages in GA eyes were assessed statistically. Increasing grades of RPE degeneration were associated with progressive loss of polarity and loss of intensity of staining of CD46, beginning with the stages that are considered normal aging (grades 0–1). Those

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GA stages with affected CD46 immunoreactivity exhibited basal laminar deposit, still-normal photoreceptors, and concomitant changes in control protein expression. Activated or anteriorly migrated RPE (grades 2–3) exhibited greatly diminished CD46. Changes in RPE CD46 expression occur early in GA, before there is evidence of morphological RPE change. At later stages of degeneration, CD46 alterations occur within a context of altered RPE polarity. These changes precede degeneration of the overlying retina and suggest that therapeutic interventions be targeted to the RPE.

Keywords

Age-related macular degeneration; geographic atrophy; complement; CD46; inflammation; immunohistochemistry; histopathology; human

1. Introduction

1.1.

Age-related macular degeneration (AMD) affects 11 million elderly individuals in the United States (Congdon et al., 2004). AMD involves four layers of the chorioretinal complex (from outer to inner): 1) the choriocapillaris; 2) Bruch's membrane (BrM), the inner wall of the choroidal vasculature and site of the hallmark extracellular lesions of AMD (drusen and basal deposits (Green and Enger, 1993; Sarks, 1976)); 3) the retinal pigment epithelium (RPE), a polarized monolayer that maintains photoreceptor health; and 4) the photoreceptors, which are ultimately affected in AMD. Choroidal neovascularization, an invasion of choriocapillaries through BrM (Grossniklaus and Green, 2004), is a sight-threatening complication in ~15% of AMD patients, treatable with intraocular injections of vascular endothelial growth factor (VEGF) inhibitors (Ciulla and Rosenfeld, 2009; Rich et al., 2006). In contrast, geographic atrophy (GA), manifesting as sharply circumscribed areas of RPE cell death across the macula, preceded by soft drusen and RPE detachments (Klein et al., 2008; Roquet et al., 2004), is a slow and currently untreatable loss of vision.

1.2.

Now that treating choroidal neovascularization is lowering the burden of vision loss from that complication of AMD, GA is receiving attention as ophthalmology's next major challenge for new therapeutic approaches (Meleth et al., 2011). Clinical trials involving immunomodulatory, anti-oxidant, retinoid-binding, and cyto-protective agents are underway. Quantifying the rate or extent of the slow outward expansion of atrophy is recognized as appropriate for tracking GA progression and monitoring efficacy of new treatments (Csaky et al., 2008; Sunness et al., 2007a) (Csaky et al., 2008; Sunness et al., 2007b). Further, contemporary clinical imaging techniques reveal the transition zone between unaffected and atrophic RPE in unprecedented detail (Bearely et al., 2009; Brar et al., 2009a; Fleckenstein et al., 2008; Fleckenstein et al., 2010). Because cells at this critical transition zone between normal and atrophic retina are likely to die and in fact may be fated for death regardless of intervention, an understanding of the molecular changes in this region is important for validating GA enlargement as a clinical endpoint. Histopathologic characterizations of this GA transition are limited (Curcio et al., 1996; Guidry et al., 2002; McLeod et al., 2009; Sarks et al., 1988), showing RPE cells of irregular shape and pigmentation, involuted choriocapillaries, loss of photoreceptors (especially rods), and, up-regulation of the intermediate filament protein vimentin in reactive, overtly altered RPE (Guidry et al., 2002). These changes are a classic RPE stress response, but the events leading to these alterations are unknown, although toxic Alu RNA has been recently implicated

(Kaneko et al., 2011). Initial changes occurring within RPE are thought to be central to AMD pathogenesis (Hogan, 1972).

1.3.

The complement system clearly is involved in the pathogenesis of AMD, with many activated fragments of the complement cascade detectable in drusen, (Anderson et al., 2002; Crabb et al., 2002; Hageman et al., 2001; Mullins et al., 2000; Wang et al., 2010b) and alterations in complement factors, both pro-inflammatory (Jakobsdottir et al., 2008; McKay et al., 2009) and regulatory, (Edwards et al., 2005; Hageman, 2005; Haines et al., 2005; Jakobsdottir et al., 2008; Klein et al., 2005; Zarepari et al., 2005) associated with disease risk. Host tissue is protected from complement by both fluid phase and membrane-bound regulators of complement activation (RCA). The former, specifically factor H, have been most widely linked to AMD risk. (Edwards et al., 2005; Hageman, 2005; Haines et al., 2005; Klein et al., 2005; Zarepari et al., 2005) The membrane-bound RCAs CD46 (also called membrane cofactor protein or MCP), CD55 (also called decay accelerating factor or DAF), and CD59 interfere with complement activation at the critical C3 step (CD46 and CD55) or prevent the actions of the membrane attack complex (CD59).

1.4.

The expression pattern of these membrane-bound RCAs has been established in the eye. Cultured human RPE cells exhibit high expression of CD59, intermediate expression of CD46, and low expression of CD55, though the latter is abundant in choroidal endothelial cells (Yang et al., 2009). Of these 3 RCA, CD46 protein was most abundant in freshly isolated human RPE cells, with CD59 protein minimally detectable and CD55 non-detectable. These latter Western blot findings agree with prior work including ours (McLaughlin et al., 2003; Vogt et al., 2006) showing that CD46 is expressed in a highly polarized fashion on the basolateral surface of the RPE. CD46 is thus the only membrane-bound RCA detected by immunohistochemistry on human RPE.

1.5.

Because of its exquisite localization on the basolateral RPE and its presence as at least the most abundant, if not only, RCA on the RPE, CD46 offers the opportunity to address several research questions regarding the RPE and GA and thus was the focus of our studies.

1.5.1.—First, we assessed potential role of CD46 in GA by examining the timing of its expression changes relative to disease progression by quantifying CD46 immunoreactivity changes across the transition from normal to GA in human donor eyes, using a semi-quantitative grading system for RPE pathology (Curcio et al., 1998; Guidry et al., 2002; Sarks, 1976; van der Schaft et al., 1992b) revised to incorporate additional intermediate stages.

1.5.2.—Second, because polarity is an essential RPE function, we provided a cellular context for the CD46 changes with additional immunolabeling studies using other polarized proteins: MCT3 (basolateral) and ezrin (apical).

1.5.3.—Third, since the RPE, photoreceptors, and choriocapillaris are a tightly integrated functional unit, we provided a global context for the RPE changes within overall chorioretinal health by assessing photoreceptor outer segment length, abundance of basal laminar deposit, and BrM calcification (Spraul and Grossniklaus, 1997a; van der Schaft et al., 1992a) within vertically oriented strips of adjoining retina and choroid. We find that

polarized protein expression and likely CD46-conferred protection is impaired remarkably early in the GA transition.

2. Methods

2.1. Donor eyes

2.1.1.—All research adhered to the tenets of the Declaration of Helsinki and IRB approval was obtained prior to initiation. Details of donors are provided in Table 1. Eleven human donor eyes with grossly visible RPE atrophy consistent with GA and without other gross chorioretinal pathology and 5 age-matched control eyes without AMD were provided by the UAB Age Related Maculopathy Histopathology Laboratory. These eyes resulted from screening post-mortem fundus appearance in non-diabetic donor eyes obtained within 6 hr post-mortem. Clinical records were obtained by follow-up with donor families and eye care providers. Globes were fixed in phosphate-buffered 4% paraformaldehyde following removal of the anterior segment and stored in 1% paraformaldehyde at 4°C (Vogt et al., 2006). Maculas were photographed using epi- and trans-illumination after removal of the vitreous.

2.2. Tissue preparation, sectioning, and staining

2.2.1.—Rectangular blocks (5 × 8 mm) of fixed tissue containing the macula were cryoprotected, embedded in sucrose OCT, frozen in liquid nitrogen, and sectioned at 10 µM (Vogt et al., 2006). Slides were selected for H&E staining from 3 separate planes through the macula. Selected planes contained at least one transition zone between normal and atrophy, on the temporal side of the macula. Since GA in several eyes included atrophy associated with the peripapillary area, a transition on the nasal side did not exist in those eyes. To permit assessment of overall chorioretinal health, adjacent slides from the 3 planes were stained with periodic acid-Schiff hematoxylin (PASH) for basement membranes and basal laminar deposit and von Kossa for calcium deposition in BrM (kits from Poly Scientific R&D Corp., Bay Shore NY).

2.3. Immunohistochemistry

2.3.1.—Three macular sections per 11 GA and 5 control eyes were analyzed for CD46 and MCT3. One section in each of 5 GA and 2 controls were analyzed for ezrin. Proteins were localized using an alkaline phosphatase Vectastain ABC Kit and a Vector Red Substrate Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions, as described (Vogt et al., 2006). Tissue was incubated overnight at 4°C with either a monoclonal antibody to CD46 (1:100, clone E4.3, Pharmingen, San Diego, CA), a rabbit polyclonal antibody to MCT3 (1:200) (Philp et al., 2003), or a mouse monoclonal to ezrin (1:100, clone 3C12, Abcam)(Kivela et al., 2000). Levamisole was added to minimize background. Slides were dehydrated through ascending alcohols and mounted with Permount (Fisher Scientific, Pittsburgh, PA). Isotype controls with equivalent concentrations of antibody (mouse IgG_{2a} for CD46 (and ezrin) and rabbit IgG for MCT3) were processed concurrently and were negative (not shown).

2.3.2.—A second, smaller section of healthy appearing retina from the same donor was included on the slide with the macular tissue section to serve as an internal positive control for protein labeling. Donor eyes that did not reveal positive staining for either protein in the healthy retinal section were excluded from analysis.

2.4. Microscopic evaluation and imaging

2.4.1.—Zones corresponding to the different RPE morphology grades of the Alabama AMD Grading System (Curcio et al., 1998; Guidry et al., 2002) were independently identified in each tissue section by 2 individuals with expertise in GA and ophthalmic pathology (CA and RWR). Slides were viewed using two research microscopes (BX51, Olympus, Center Valley PA and Eclipse 80i, Nikon, Melville, NY). Sections were read from temporal to nasal (i.e., across the fovea and towards the optic nerve head). To permit determination of zone lengths, zone boundaries were recorded as lengths along BrM in the PASH-stained section using a stepping-motor stage with digital read-out (Ludl, Hawthorne NY) on the Eclipse microscope. Chorioretinal health was assessed by semi-quantitative measures of photoreceptor outer segment length (normal, short, or absent, relative to uninvolved retina on the same section) and basal laminar deposit thickness (none, patchy, $<1/2$ normal RPE height, $>1/2$ normal RPE height) for each zone in the PASH-stained sections. To evaluate BrM calcium deposition, the proportion of BrM containing black von Kossa precipitate was expressed as a percent of total BrM length and thickness ($< \text{BrM}$, $=\text{BrM}$, $>\text{BrM}$) for each zone. For CD46, MCT3, and ezrin immunoreactivity, intensity of staining ($<\text{normal}$, normal, $>\text{normal}$) was determined relative to normal RPE on the same slide. Location of staining on individual RPE cells was also recorded. For CD46 and MCT3, location was characterized as “basolateral” or “not basolateral” while for ezrin, location was “apical” or “not apical” with an additional assessment included for the status of delicate apical processes, which could be pulled into an upright position by retinal detachment in some cases or compacted into a layer on the RPE apical surface by others. Information was recorded at the time of microscopy in a custom database (FileMaker, Santa Clara CA).

2.4.2.—Differential interference contrast optics in bright field was used on the Olympus BX51 microscope and digital images captured using an Olympus Qcolor 5 camera (Olympus, Center Valley, PA) and Qcapture software (Burnaby, Canada). Images were assembled into a composite without enhancement (Adobe Photoshop CS2, San Jose CA).

2.5. Data analysis and statistics

2.5.1.—The relationship of chorioretinal health and immunoreactivity to RPE pathology grade within and between GA and control eyes was evaluated by pooling semi-quantitative values for each parameter across eyes (1132 graded RPE zones total). Analysis incorporated mixed statistical models to account for clustering of zones within individual eyes.

2.6. SNP genotyping

2.6.1.—Genomic DNA was isolated from peripheral retina preserved in 1% paraformaldehyde using the Recover All Total Nucleic Acid kit (Ambion Inc., Austin TX) according to manufacturer’s instructions. Briefly, tissue was dehydrated in a series of increasing ethanol concentrations ranging from 30% to 100% and then digested with protease at 50°C for 48 hr. DNA concentration (260/280 ratio = 1.8) was determined spectrophotometrically (NanoDrop™ 1000 Thermo Fisher Scientific, Wilmington, DE). Genotyping for complement component 2 (C2, rs9332739), complement factor B (CFB, rs4151667), and age-related maculopathy susceptibility protein 2 (ARMS2, rs10490924) was performed using Taqman single nucleotide polymorphism (SNP) Genotyping Assays. Complement factor H (CFH, rs1061170) and HtrA serine peptidase 1 (HTRA1, rs11200638) polymorphisms were determined using Custom Taqman SNP Genotyping Assays (Applied Biosystems, Foster City, CA). Product was amplified using a PTC 200 thermocycler PCR system (MJ Research, Reno, NV).

3. Results

3.1. Patients and eyes, sampling methods, criteria

3.1.1.—Information about study eyes is summarized in Table 1. Eleven GA eyes came from 8 women and 3 men (age 87.0 ± 4.1 yr). Five control eyes came from 3 women and 2 men (age 84.0 ± 8.9 yr). All donors were Caucasian. Available eye health history for 4 of the GA eyes indicated a clinical presentation of AMD at 1.8 to 87.8 months prior to donor death, with visual acuity in these eyes ranging from mildly to severely impaired (Table 1). To characterize the genetic risk profile of each donor, the genotype for several known enhancing and protective genetic states were determined (Table 1). Analysis of risk and protective genotypes for C2, CFB, CFH, HTRA1, and ARMS2 showed only that a greater number of individuals with ARMS2 risk alleles were among GA donors.

3.1.2.—For GA eyes, large areas of RPE atrophy consistent with that diagnosis were evident in the fundi of preserved eyes (Figure 1). The margin between normal and atrophic RPE assumed one of two forms, smooth (Figure 1A) or lobulated (Figure 1B)(Table 1) (Brar et al., 2009b), the latter featuring multiple circular atrophic areas with intervening RPE islands. Some eyes had choroidal atrophy within the area of geographic RPE atrophy (Figure 1A) and drusen were occasionally visible (Figure 1A). An age-related peripapillary chorioretinal degeneration similar to AMD (Curcio et al., 2000; Jonas et al., 1989) surrounded the nerve head completely (n=5) or partly (n=4) in most samples.

3.2. RPE Staging

3.2.1.—A representative section divided into zones of RPE morphology is shown in Figure 2, and examples of each morphology stage for each stain are shown in Figure 3. Grades 0 (uniform pigmentation and morphology, Figure 3, **column 0**) and 1 (non-uniform but still epithelioid pigmentation and morphology, Figure 3, **column 1**) are considered normal aging (Sarks et al., 2007; Sarks, 1976) and will therefore be collectively called normal. Grades 2A (heaping of RPE cells with anterior migration into the sub-retinal space, Figure 3, **column 2A**), 2B (basal migration into basal deposits, Figure 3, **column 2B**), and 2L (leaflets of RPE cells, Figure 3, **column 2L**) indicate an RPE that is attached only partly to BrM or basal deposits. Grade 3 (Figure 3, **column 3**) reveals anterior migration of pigmented cells across the sub-retinal space through the external limiting membrane and into the neurosensory retina. The fate of these migrating cells is not fully known but melanin and lipofuscin granules can be found interspersed among photoreceptor axons and Müller glial cells in the Henle fiber layer (not shown), suggesting clearance by resident retinal phagocytes. Atrophic areas include Grade 4 (loss of pigmented cells with persisting basal deposits, Figure 3, **column 4**) and 5 (loss of both pigmented cells and basal laminar deposit, not shown).

3.3. RPE Status and Chorioretinal Health

3.3.1.—The photoreceptors, RPE, and BrM form a highly integrated physiological unit. We evaluated the characteristics of the photoreceptor outer segments and BrM in relation to the adjacent zones of RPE change. A total of 1132 RPE zones were characterized, summarized in Table 2. Control eyes contained mainly long zones of grade 0 and 1, with shorter zones of grades 4 and 5, the latter found exclusively in the peripapillary region. In GA eyes, zones of grade 0 and 1 were shorter than in control eyes, with the addition of numerous short zones of grades 2 to 3. In most eyes, zones at the uninvolved temporal end of sections were rated as grades 0 and 1 while the higher grades were located next to the macular atrophic area. Grades 2A and 2L were on average similar in length, and grade 2B was shorter (Table 2). Grades 2B and 2L were always closer to the atrophic area than 2A. In eyes with lobulated GA margins, the small RPE islands between atrophic areas were exclusively grades 2A, 2B, 2L, or 3. The transition from normal to atrophic RPE typically included 1 to 3 zones of

varying grades. Of 166 transitions from 0/1 (normal) to 4/5 (atrophic) observed in GA eyes, 24 (14.5%) passed directly from normal to atrophic, 97 (58.4%) included one intervening zone of grades 2 to 3 (median length, 1.188 mm), and 45 (27.1%) included 2 or more zones of grades 2 to 3 (median length, 2.371 mm).

3.3.2.—Photoreceptor outer segments (OS) shorten early in the course of all degenerations and injuries affecting these cells (Ripps, 1982). We characterized the length of the OS overlying each RPE zone as either normal (Figure 2B), shortened (Figure 2C), or absent (Figure 2G). In control eyes the OS were absent only adjacent to peripapillary RPE atrophy. In GA eyes, the OS were absent in many zones of grade 2A and higher. Significant changes in OS length were found as RPE atrophy progressed from grades 0 to 5 but not in the early transition between 0 and 1 (Table 3).

3.3.3.—Basal laminar deposit (BlamD) is extracellular basement membrane material formed by the RPE, the abundance of which is associated with AMD severity (Sarks et al., 2007; Spraul and Grossniklaus, 1997a). We characterized the presence of BlamD as patchy (Figure 2C), thin continuous (Figure 2E), or thick continuous (Figure 2G). In control eyes BlamD was minimal and almost exclusively patchy. In GA eyes BlamD was significantly more prominent early in the progression between RPE grades 0 and 1 (Table 3).

3.3.4.—Calcification of BrM is associated with risk for choroidal neovascularization (Spraul and Grossniklaus, 1997b). We assessed the degree of calcification of BrM within each RPE zone in von Kossa-stained sections. The percentage of BrM length and the proportion of BrM thickness stained, characterized as less than full thickness (<BrM, Figure 2K) or greater than full thickness (>BrM, Figure 2M), were recorded. In control eyes, BrM calcification was minimal except in the peripapillary region and showed no relationship to RPE grade (Table 3). In GA eyes, calcification of BrM, both in terms of length and thickness, increased with RPE grade, with significant differences among grades 0 and 5 but not between 0 and 1 (Table 3).

3.4.

In summary, all three laminar health indices (photoreceptor outer segment length, BlamD, and BrM calcification) worsened with advancing RPE morphology grade. The index signaling the earliest difference between GA and control eyes and between grades 0 and 1 in GA eyes was BlamD.

3.5. CD46 immunoreactivity: intensity and localization

3.5.1.—CD46 is a type I transmembrane glycoprotein expressed on all nucleated cells with four commonly expressed isoforms produced by alternative splicing of its gene. CD46 contains four extracellular short consensus repeats, a transmembrane region, and one of two possible alternatively spliced cytoplasmic tails, both of which contain signalling motifs that mediate distinct cellular functions. CD46 is the only ubiquitously expressed cofactor for the Factor I mediated cleavage of C3b and C4b (Reviewed in (Cardone et al., 2011)).

3.5.2.—CD46 was readily recognized by the specific and exclusively basolateral location of red reaction product in grade 0 zones of control and GA eyes (Figure 3R) (Vogt et al., 2006). With increasing severity of RPE change, immunoreactivity became less intense (Figure 3S–W). Little CD46 was visible in zones at grade 2L and higher. Further, the location of immunoreactivity departed from basolateral restriction at early grades of RPE change, becoming diffuse and non-polarized (Figure 3T–V). Statistical analysis of CD46 immunoreactivity associated with different RPE grades of GA or control eyes, and between the healthier RPE grades (0, 1) of both groups is provided in Table 4. Alteration from

normal in intensity and location were significantly associated with increasing severity of RPE grade in GA, with borderline significant differences between CD46 intensities at grades 0 and 1. Graphical analysis (Figure 4) shows the reduction in CD46 staining intensity and basolateral localization with the progression of RPE alteration across GA lesions.

3.6. Analysis of other polarized proteins (MCT3 and ezrin): intensity and location

3.6.1.—To determine if altered CD46 expression is due to global changes in polarized protein expression unrelated to complement, we assessed immunoreactivity for MCT3, a basolaterally expressed transporter, and ezrin, an apically expressed actin-associated protein.

3.6.2.—The MCTs are members of the SLC16A gene family of transporters and several MCT isoforms are expressed in the mammalian eye. MCT3 (Slc16a8) is preferentially expressed at high levels in the differentiated RPE cells where it is polarized to the RPE basolateral membrane (Longbottom et al., 2009; Philp et al., 2001).

3.6.3.—Overall, MCT3 exhibited more intense staining in grade 0 of control and GA eyes (Figure 3Z) than CD46. MCT3 was present diffusely and exclusively on the inner leaflet of grade 2L RPE cells (Figure 3DD). MCT3 was not detected in any grade 3 cells within the neurosensory retina. MCT3 was detected extracellularly (Figure 3BB,CC), even within deposits persisting after complete RPE atrophy (Figure 3FF), consistent with others findings about membrane-bound proteins of basolateral RPE origin (Gouras et al., 2009; Johnson et al., 2001). Statistical and graphical analysis of MCT3 immunoreactivity (Table 4, Figure 4) showed that the decline with advancing pathology was consistent with that seen with CD46. At the earliest stages of RPE change within GA eyes, MCT3 intensity differs significantly between grades 0 and 1 while location changes are of borderline significance at that stage.

3.6.4.—The ezrin/ radixin/ moesin (ERM) protein family is part of the cortical cytoskeleton and provides architectural and/or regulatory contribution to epithelial actin cytoskeleton. Ezrin is localized in the long apical microvilli of RPE cells that invest outer segment tips and participate in phagocytosis, serving as an epithelial marker and a morphogenetic agent in development (Bonilha, 2007).

3.6.5.—Ezrin immunoreactivity was decreased in the macula of GA eyes, roughly in relation to worsening RPE pathology (Figure 5). The small sample of eyes available for ezrin staining precludes assessing the stage of change to the same degree possible for CD46 and MCT3. Changes in ezrin localization were consistent with those seen for MCT3, i.e., labeling was not confined to the typical apical domain, only the inner leaflet of stage 2L showed orthotopic staining, and extracellular labeling was detected. Some cells sloughed into the sub-retinal space (2A) exhibited diffusely distributed ezrin immunoreactivity without detectable apical processes.

4. Discussion

4.1.

The current study includes, to our knowledge, the largest group to date of exclusively GA eyes examined with immunohistochemical methods and the first to focus on RPE health and the transition between normal and atrophic cells. We found alterations in RPE expression of complement regulator CD46, at least one other RPE specific basolateral protein (MCT3) and an apical protein (ezrin). Strikingly, these alterations are apparent at the earliest stages of GA (stage 1), prior to frank atrophy or even clinically detectable change. Eyes with GA exhibited less intense CD46 staining at healthier RPE grades (0–1) than control eyes, suggesting altered RPE cell health even in cells with near normal morphology and in

advance of pathology in adjacent photoreceptors or BrM. Our results have significance for understanding the role of complement, the status of polarized RPE functions, and the timeline of disease progression across the GA transition.

4.2. Implications of findings for role of complement in AMD

4.2.1.—Despite multiple theories, the specific inciting event(s) leading to RPE degeneration and the development of AMD are unknown. One potential stressor is chronic inflammation, possibly evoked by lipid hydroperoxides on lipoproteins deposited with age in BrM (Curcio et al., 2009; Spaide et al., 2003) or shed cellular debris (Hageman et al., 2001). Complement is continually activated in the serum at a low level, a process known as tick over activation. If not regulated, then this low level activation is amplified and clinically significant levels of activated complement components are created. It is thus possible that a defect in complement regulation by any RCA could result in the chronic inflammation thought involved in AMD pathogenesis. This concept is supported by both genetic and tissue-based studies. A tyrosine-402 to histidine-402 protein polymorphism in the RCA Factor H is associated with a significantly increased AMD risk [Edwards, 2005 #3030; Hageman, 2005 #3027; Haines, 2005 #3029; Klein, 2005 #3028]. Associations with other complement component genes have also been found, including factor B, C2, and C3. (Gold et al., 2006) (Maller et al., 2007). Drusen contain complement components C1q, C3, C5, and C5b-9 (Anderson et al., 2002; Crabb et al., 2002; Hageman et al., 2001; Mullins et al., 2000; Wang et al., 2010b).

4.2.2.—Based on the hypothesis that reduced CD46 on the RPE is detrimental to the RPE, we sought to determine if changes in the presence of CD46 on the RPE were associated with early stage AMD. We found alterations in RPE CD46 expression progressively increase as RPE morphology deteriorates in the transition from normal to GA. Of greatest significance, changes in CD46 expression occur even on grade 1 RPE cells, which exhibit minimal morphological alterations. Whether these CD46 changes indicate that a defect in complement protection is an initiating factor in GA or another manifestation of an already dysfunctional RPE remains to be determined. Either of these scenarios could lead to the extracellular complement deposition documented in AMD, as both result in reduced CD46 expression on the RPE. Others have shown that CD46 on the RPE interacts with β -1 integrin, suggesting a role in basement membrane adhesion (McLaughlin et al., 2003). A progressive loss of this interaction could explain the release of RPE cells from BrM in grades 2 and 3. The classic description of complement actions includes nonspecific targeting of cells for phagocytosis, induction of inflammation via anaphylatoxin production, and assembly of the membrane attack complex, a pore forming structure that inserts into cell membranes to disrupt homeostasis (Walport, 2001a, b). However, features of this acute phase reaction, such as an influx of neutrophils and macrophages (at least in large number), and lysis of RPE, were not detectable in our sample, suggesting that complement's involvement in GA is not in the "classical" sense, but rather is likely involved in induction of chronic inflammation.

4.3. Implications of findings for RPE and its polarized functions

4.3.1.—We provided the first in situ evidence regarding RPE polarity status in AMD eyes, with the finding that stage-specific loss of CD46 occurs in the setting of decreased expression of two other highly polarized RPE proteins. Part of the outer-blood retinal barrier, the RPE provides essential services to both photoreceptors and choriocapillaris, and like other epithelia, it is structurally and functionally polarized (Strauss, 2005). The RPE polarity program includes mechanisms for directional sorting and delivery of bio-molecules and maintenance of apical and basolateral domain-identity. The latter, in turn, involves the circumferential belt of tight junctions that provide a fence between those two domains and

helps organize intracellular secretory, endocytotic, and cytoskeletal pathways (Tanos and Rodriguez-Boulan, 2008). miR-204 and Mir-211 expression are essential for maintaining differentiated properties of the RPE including junctional proteins that maintain trans-epithelial resistance (Wang et al., 2010a). Trans-epithelial transport of lactate from aerobic glycolysis (Wang et al., 1997; Winkler et al., 1997) is facilitated by monocarboxylate transporters (MCTs) in apical and basolateral RPE membranes (Philp et al., 2003). MCT3 expression is developmentally regulated, making it a marker for differentiated RPE cells, and loss of MCT3 occurs rapidly in mechanically wounded monolayers of human fetal RPE. (Gallagher-Colombo et al., 2010). Ezrin is present throughout the entire length of RPE microvilli, which play essential roles in phagocytosis and retinoid processing (Bonilha et al., 2004; Steinberg et al., 1977). As apical processes have been observed in early AMD eyes (Curcio et al., 1996), their loss in GA is a novel observation, warranting additional samples for more detailed characterization.

4.3.2.—We propose that the observed changes in CD46, MCT3, and ezrin expression collectively serve as bellwethers for other key RPE functions that may also be impaired in AMD eyes. These include orchestration of chemokine release (Shi et al., 2008), secretion of angiogenic VEGF and angiostatic PEDF (Sonoda et al., 2010), and expression of junctional complexes (Economopoulou et al., 2009), all of which are dramatically less efficient in non-polarized cells (Sonoda et al., 2010).

4.4. Implications of approach for time line of GA progression

4.4.1.—Our RPE-based grading system exploits the layered organization of the retina and choroid, using spatial localization as a surrogate for time of disease progression. We refined existing histopathologic stagings of AMD severity (Curcio et al., 1998; Curcio et al., 2000; Guidry et al., 2002; Sarks, 1976; van der Schaft et al., 1992b) to include several RPE morphologies (grades 2A, 2B, 2L) preceding cell death (grade 4). Grades were based on a characteristic RPE stress response described in multiple conditions (Anderson et al., 2010; Capon et al., 1989; Hahn, 2004; Hilton, 1974; Kuntz et al., 1996; Lopez et al., 1995; Zhao et al., 2011). These RPE grades *do not* represent snapshots of a linear process in time. Early reactive Grade 2A cells are plausible direct precursors of grade 3 cells, but grades 2B and 2L appear to represent alternative routes to RPE death. Of note, in clinical descriptions of the GA transition zone using spectral domain OCT (SD-OCT), the hyper-reflective band commonly interpreted as RPE-BrM shows irregularities, elevations, double layers, and associated clumps in the subretinal space and neurosensory retina (Fleckenstein et al., 2008), remarkably similar to our grades 2–3. Further, over time these areas lose reflectivity and eventually fade to become newly formed atrophy (Fleckenstein et al., 2010), consistent with our histological interpretations.

4.4.2.—We found that reduced CD46 and MCT3 immunoreactivity and basal deposit accumulation occurred early in GA disease process, when RPE morphology was minimally affected at grade 1, whereas photoreceptor degeneration was not reliably detected until the RPE was more seriously affected at grade 2A. Although patchy BlamD is considered normal aging (Bressler et al., 2006), it begins a continuum that ends with the abundant BlamD associated with AMD (Spraul and Grossniklaus, 1997a). These changes should be interpreted in the context of normal aging, as, rod photoreceptors in the macula die slowly throughout adulthood and RPE numbers remain stable (Curcio et al., 1993; Del Priore et al., 2002). In contrast, in GA loss of RPE polarity and deposition of BlamD together suggest that RPE degeneration precedes that of the photoreceptors, at least as detectable by light microscopy. Further, current evidence suggests that RPE dysfunction also precedes secondary choriocapillaris loss in GA (McLeod et al., 2009). This implies that therapies and interventions to save outer retinal functions are best directed at the RPE.

4.5.

In summary, we have shown that RPE expression of the complement regulator CD46 at the very earliest stages of AMD. This alteration consists of a loss of intensity of staining as well as alterations in the polarized location of staining, the latter associated with more advanced stages of RPE degeneration. These changes parallel those seen in other polarized proteins not associated with complement (MCT3 and ezrin). The current study is limited by its use of immunohistochemical methods to detect protein presence and semi-quantify protein levels as well as by a limitation in the number of stains that could be performed due to tissue availability. Thus a characterization of whether alterations in complement activation status or fragment deposition were present concurrent with regulator alteration was not performed. Nonetheless, the findings presented are intriguing in their demonstrating RPE protein alterations at the earliest stages of GA, before clinically apparent changes are evident.

Highlights

- We examine expression of complement regulator CD46 on the RPE in geographic atrophy
- CD46 expression is compared to control polarized proteins MCT3 and ezrin
- CD46 expression is altered at the earliest stage of geographic atrophy
- This alteration occurs before clinically detectable changes are evident

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Figure 1. Post-mortem fundus appearance in donor eyes with geographic atrophy
 Maculas with anterior segments removed were photographed with trans- and epi-illumination (shown as right eyes). Star indicates optic nerve head. Large retinal vessels are dark red (especially in A). The choroidal circulation, beneath the retina, is best visible in areas where the RPE is atrophic, and the emptied large vessels contrast with the intervening pigmented stroma. Bar = 1 mm. **A.** Geographic atrophy with a smooth border. White arrowhead, border; white arrow, peripapillary atrophy border; black arrowhead, border of choroidal atrophy. (ID #2000051L, 93 yr, male) **B.** Geographic atrophy with a lobulated border. White arrowhead, lobulated GA border; black arrows, drusen. (ID# 2000028L, 91

yr, female) Green line demonstrates how histological sections transitioned from unaffected to atrophic areas.

Figure 2. Section of a GA macula demonstrating assignment of RPE pathology zones. Section is stained with periodic acid Schiff-hematoxylin and is located superior to the fovea. Optic nerve and peripapillary terminus of BrM is at the right. Zones of different grades of RPE morphology are indicated. In the evaluation of RPE morphology, sections were read from the uninvolved temporal end (at the left), and new zones were assigned at the first encounter with new pathology.

Figure 3. Grades of RPE change and associated staining patterns in geographic atrophy (for a 2-page spread)

The first column shows control eyes. Other columns show different stains at each pathology grade from different GA eyes. Grades of worsening pathology are arranged across rows. Bright field and differential interference contrast microscopy. **Bars, 50 μ m. Periodic acid Schiff hematoxylin (PASH).** **A.** Control, grade 0. **B.** Grade 0, normal RPE morphology. IS, inner segments of photoreceptors; OS, outer segments of photoreceptors; RPE, retinal pigment epithelium; Ch, choroid. **C.** Grade 1, non-uniform RPE, patches of basal deposits. **D.** Grade 2A, heaped and sloughed RPE, BrM (bracketed) with basal laminar deposit on inner aspect. **E.** Grade 2B, RPE cellular material shed into basal deposits (star). **F.** Grade 2L, RPE forms 2 leaflets. **G.** RPE (arrow) invading neurosensory retina. **H.** Grade 4, RPE or photoreceptors absent, deposits present. Visible retinal cells are in the inner nuclear layer. **Von Kossa stain for calcium (VK, black)** reveals compacted laminae within BrM (**I-M**) and globular particulates within BrM (**N**), cells (**I, M**) or basal deposits (**M**). **Q, R. CD46 immunoreactivity (red)** localizes to the RPE basolateral aspect (arrowhead). **S, T, U.** CD46 immunoreactivity is lighter and not limited to the basolateral aspect. **V, W, X.** CD46 immunoreactivity is undetectable. **Y, Z. MCT3 immunoreactivity (red)** localizes to the RPE basolateral aspect (arrowhead). **AA, BB, CC.** MCT3 immunoreactivity is lighter and not limited to the basolateral aspect. **DD.** MCT3 immunoreactivity is largely, but not exclusively localized to the basolateral aspect of the inner (upper) leaflet of RPE cells. None is present in the outer (lower) leaflet. **EE, FF.** Pale MCT3 immunoreactivity is present in RPE and in basal deposits (bracketed by carets in CC), the latter persisting after cells disappeared.

Figure 4. Protein expression by RPE grade in GA eyes

Data for intensity and location of immunoreactivity are displayed as a percentage of total zones rated (1,132) pooled across all GA eyes (Table 2). The total number of zones per grade is shown at the base of each grade's bar. Normal is referenced to the staining characteristics of RPE at grade 0 in periphery of GA eyes. CD46 and MCT3 immunoreactivity diminishes in intensity and becomes progressively less polarized, in association with worsening RPE morphology. MCT3 immunoreactivity is apparent at higher grades than CD46. Basolateral indicates that staining is confined to the basolateral surface. The differences between grades 0 and 1 are significant (see Table 3) Not basolateral indicates that staining was not so confined. Legend: BL, basolateral.

Figure 5. Ezrin localization in unaffected periphery (A) and affected macula (B) of geographic atrophy eye

Immunoreactivity is red. Differential interference contrast microscopy. **A.** RPE apical processes are upright and intensely immunoreactive after detachment of neurosensory retina. **B.** Light extracellular immunoreactivity and a diffusely labeled sloughed RPE cell in a grade 2A area. Arrows delimit BlamD. ELM, external limiting membrane; IS, inner segments; OS, outer segments; RPE, retinal pigment epithelium; BrM, Bruch's membrane; ChC, choriocapillaris.

Table 1

Donor Eyes

SingleID	Group	Case #	Age (yr)	Gender	Clinic information			Genotyping results					GA margin
					LastExam (mo)	Macular Hx	VAcc	Protective allele		Risk allele			
								C2 ¹	CFB ²	CFH ³	HTRA1 ⁴	ARMS2 ⁵	
2004014L	GA	1	81	F	1.8	Dry ARMD	2	GC	AA	T _C	AA	T _G	lobulated
2000012L		2	82	M	n.a.	n.a.	n.a.	GC	TT	C _C	AA	T _T	smooth
2004061L		3	83	F	n.a.	n.a.	n.a.	GG	TT	T _C	GG	T _G	n.d
2000067L		4	84	F	n.a.	n.a.	n.a.	GG	TT	C _C	AA	T _G	lobulated
2000020L		5	85	F	8.4	Dry ARMD	4	n.a.	AA	C _C	AA	GG	smooth
2005025L		6	88	F	41.2	Other	3	GG	TT	T _C	AG	T _G	smooth
2002046L		7	89	M	33.2	CNV/ARMD	5	GG	AT	TT	AG	T _T	lobulated
2002095L		8	90	F	n.a.	n.a.	n.a.	GG	AA	C _C	AA	GG	lobulated
2000028L		9	91	F	n.a.	n.a.	n.a.	GG	TT	T _C	AG	T _G	lobulated
2004053L		10	91	F	87.8	Dry ARMD	3	GC	AT	T _C	GG	T _G	lobulated
2000051L		11	93	M	n.a.	n.a.	n.a.	GC	TT	C _C	AA	T _G	no record
2004008L	Control	12	75	M	14.3	Normal	1	GG	TT	C _C	GG	GG	n.a.
2002097L		13	85	F	n.a.	n.a.	n.a.	GG	TT	T _C	AA	GG	n.a.
2003107L		14	92	F	n.a.	n.a.	n.a.	GC	AT	C _C	AA	GG	n.a.
2004054L		15	92	M	n.a.	n.a.	n.a.	GG	TT	T _C	GG	GG	n.a.
2002091R		16	94	F	n.a.	n.a.	n.a.	GG	TT	T _C	AG	T _G	n.a.

All donors were Caucasian. **n.d.**, not determinable from photographs; **n.a.**, not applicable/available.

VA_{cc}, corrected visual acuity; **1**=20/20 or better; **2**=20/25 – 20/40; **3**= 20/50 – 20/200; **4**=20/300 – 20/1600; **5**= hand movement – light perception.

- ¹. Complement component 2, SNP rs 9332739, Protective allele C is bold and italicized.
- ². Complement factor B, SNP rs 4151667, Protective allele A is bold and italicized.
- ³. Complement factor H, SNP rs1061170 (Y402H). Risk allele C is underlined.
- ⁴. HTRA1 serine peptidase 1, SNP, rs11200638 (–625G/A). Risk allele A is underlined.
- ⁵. ARSM2, SNP rs 10490924 (A69S). Risk allele T is underlined.

Table 2

Zones of RPE morphology

GA	RPE Grade									
	0	1	2A	2B	2L	3	4	5		
# zones	75	180	185	22	88	107	193	73		
% of total zones	8.1%	19.5%	20.0%	2.4%	9.5%	11.6%	20.9%	7.9%		
Mean length, mm	1.783	1.376	1.238	0.825	1.262	0.731	1.238	1.284		
Maximum length, mm	4.206	4.479	4.622	4.473	4.334	3.771	4.838	4.334		
% of total length analyzed	11.6%	21.5%	19.9%	1.6%	9.7%	6.8%	20.8%	8.1%		
Control	0	1	2A	2B	2L	3	4	5		
# zones	53	31	9	0	0	1	33	8		
% of total zones	39.3%	23.0%	6.7%	0.0%	0.0%	0.7%	24.4%	5.9%		
Mean length, mm	5.420	1.131	0.330	0.000	0.000	0.549	0.305	0.231		
Maximum length, mm	8.080	6.453	0.626	0.000	0.000	0.549	0.766	0.360		
% of total length analyzed	85.0%	10.4%	0.9%	0.0%	0.0%	0.2%	3.0%	0.5%		

Legend: RPE, retinal pigment epithelium

Table 3

Chorioretinal health in relation to RPE grade

Feature	Group	Pattern	RPE Grade					Within group P value					Between group P value		
			0	1	2A	2B	2L	3	4	5	Total	Grades 0–5	Grades 0–1	Grades 0–5	Grades 0–1
Outer Segments	GA	Absent	0	5	24	4	15	33	49	20	150				
		Short	1	6	11	4	7	6	0	0	35				
		Normal	12	27	9	1	0	0	2	0	51	<0.0001	0.2603		
		Total	13	38	44	9	22	39	51	20	236				
	Control	Absent	0	0	1	0	0	0	4	2	7				0.1363
Short		1	2	1	0	0	0	0	0	4					
Normal		20	9	4	0	0	0	0	0	33	<0.0001	0.2234			
Total		21	11	6	0	0	0	4	2	44					
BlamD	GA	None	11	11	3	0	1	2	2	12	42				
		Patchy	2	15	14	1	14	3	15	3	67				
		<1/2	0	10	15	6	2	20	23	1	77	<0.0001	0.0059		
		>1/2	0	2	12	2	5	14	8	0	43				
	Total	13	38	44	9	22	39	48	16	229			<0.0001	0.0088	
Calcification (%BrM Length)	GA	None	12	5	3	0	0	0	1	2	23				
		Patchy	9	5	2	0	0	0	1	0	17				
		<1/2	0	1	0	0	0	0	1	0	2	0.2309	0.3606		
		>1/2	0	0	1	0	0	0	0	0	1				
	Total	21	11	6	0	0	0	3	2	43					
Control	Mean%	36	43	50	16	16	64	60	68						
	SD	35	38	39	28	23	38	38	32				0.49		
	Total	20	75	85	13	23	40	75	39	370			0.4814	0.0254	
	Mean %	13	35	40	0	30	0	42	20						
	SD	23	37	14	0	----	0	18	0				0.05		

Legend: RPE, retinal pigment epithelium; GA, geographic atrophy; BlamD, basal laminar deposit; BrM, Bruch's membrane

“Total” refers to total number of observations in each grade.

Note: see Methods for description of grades. $<1/2$, $>1/2$ is reference to the typical height of normal RPE.

Table 4

Association of CD46 and MCT3 staining features with RPE grade

Feature	Group	Pattern	RPE Grade										Within group <i>P</i> value		Between group <i>P</i> value	
			0	1	2A	2B	2L	3	4	5	Total	Grades 0–5	Grades 0–1	Grades 0–5	Grades 0–1	
CD46 Intensity	GA	Absent	6	54	50	12	16	29	91	32	290					
		<Normal	15	55	47	5	9	7	7	3	148					
		=Normal	8	15	3	3	0	0	1	0	30	<0.0001		0.06		
		>Normal	0	1	0	0	0	0	0	0	1					
		Total	29	125	100	20	25	36	99	35	469			<0.0001	0.07	
	Control	Absent	2	3	3	0	0	0	5	2	15					
	<Normal	14	10	0	0	0	0	2	0	26						
	=Normal	4	3	0	0	0	0	0	0	7	0.0061		0.757			
	Total	20	16	3	0	0	0	7	2	48						
CD46 Location	GA	BL	20	59	38	5	4	3	5	0	134					
		Not BL	3	25	27	4	15	18	39	17	148	<0.0001		0.11		
		Total	23	84	65	9	19	21	44	17	282					
		BL	16	11	0	0	0	0	0	0	27					
		Not BL	2	4	2	0	0	0	4	1	13	0.0012		0.26		
	Control	Total	18	15	2	0	0	0	4	1	40					
MCT3 Intensity	GA	Absent	0	16	15	2	1	12	72	40	158					
		<Normal	4	56	63	11	24	26	11	1	196					
		=Normal	24	40	22	4	2	2	0	0	94	<0.0001		<0.0001		
		>Normal	0	0	2	0	0	0	0	0	2					
		Total	28	112	102	17	27	40	83	41	450			<0.0001	<0.0001	
	Control	Absent	0	2	0	0	0	0	6	2	10					
	<Normal	7	8	0	0	0	1	3	0	19	<0.0001		0.1			
	=Normal	24	12	0	0	0	0	0	0	36						

Feature	Group	Pattern	RPE Grade										Within group <i>P</i> value		Between group <i>P</i> value	
			0	1	2A	2B	2L	3	4	5	Total	Grades 0–5	Grades 0–1	Grades 0–5	Grades 0–1	
MCT3 Location	GA	Total	31	22	0	0	0	1	9	2	65					
		BL	18	44	36	7	6	6	3	0	120					
		Not BL	9	52	59	9	20	29	52	21	251	<0.0001	0.06			
	Total	27	96	95	16	26	35	55	21	371			<0.0001	0.02		
	Control	BL	11	4	0	0	0	0	0	0	15					
		Not BL	20	17	0	0	0	1	7	0	45	0.1871	0.2			
Total		31	21	0	0	0	1	7	0	60						

Note: see Methods for description of grades. BL, basolateral.
There were no instances of staining that were > Normal in the control eyes.
“Total” refers to total number of observati in each grade.