Complement Component C5a Activates ICAM-1 Expression on Human Choroidal Endothelial Cells


PURPOSE. The complement system plays a crucial role in the progression of age-related macular degeneration (AMD). In this study, the authors sought to evaluate the pathophysiologic roles of complement components C3α and C5α in the human choroid in AMD.

METHODS. Human RPE/choroid was assayed for the presence of C3α and C5α receptors (C3αR and C5αR) using RT-PCR and immunohistochemistry. Choroidal endothelial cell migration and proliferation were evaluated in the presence of C5α. Organ cultures of human choroid were incubated in C5a or bovine serum albumin (BSA) followed by quantitative immunohistochemistry and quantitative PCR for ICAM-1. AMD patients and controls were genotyped at SNPs in the C5R1 and C3AR1 genes.

RESULTS. C5αR, but not C3αR, was detected in human choroid. C5α did not promote endothelial cell migration or proliferation. However, choriocapillaris endothelial cells in organ culture responded to C5α by increasing ICAM-1 mRNA and protein. No significant association of SNP genotypes was detected in AMD patients at the C3AR1 and C5R1 genes.

CONCLUSIONS. The generation of C5α peptides may lead to activation of choriocapillaris endothelial cells in AMD. Activation of the choroidal endothelium may affect the progression of AMD by recruitment of monocytes, leading to additional sequelae of AMD pathogenesis.

A ge-related macular degeneration (AMD) is a potentially blinding eye disease that affects more than one-third of the elderly population to some degree. This disease affects the macula, the central region of the retina responsible for high-acuity visual tasks such as reading and driving. AMD is commonly divided into two classes. The first is atrophic, or dry, AMD characterized by the development of sub-retinal pigment epithelial deposits known as drusen and atrophy of the retinal pigment epithelium (RPE) that can lead to severe retinal degeneration. The second class is called exudative, or wet, AMD because of the extracellular exudation or fluid deposition that results from choroidal neovascularization. Exudative AMD is synonymous with the prior development of choroidal neovascularization, disciform (disc-shaped) scarring in the subretinal space, and detachment of the RPE, which leads to vision loss. Exudative AMD typically occurs later in the pathogenesis of the disease and results in an acute and often profound reduction in visual acuity.

The complement system is part of the innate immune system and is composed of a diverse array of structural proteins and enzymes that promote or inhibit complement activation, which, in turn, leads to target cell chemotaxis, cellular activation, lysis, or apoptosis. Regardless of how they are initiated, complement cascades merge at the proteolytic activation of protein C3. Cleavage of C5 leads to the formation of C5a and C5b. C5b functions cooperatively as a C5 convertase, which cleaves C5 into C5a and C5b. If not inactivated or quenched, the C5b fragment combines with other complement cascade proteins to form the membrane attack complex (C5b-9). The membrane attack complex forms on the surface of target cells, causing their destruction by forming membrane-spanning pores. In addition to the membrane attack complex, complement activation results in the formation of anaphylatoxins C3a and C5a. These N-terminal fragments of complement proteins are capable of promoting inflammation, and an increase in anaphylatoxin production may cause prolonged cell activation and an increased level of local inflammation, which may lead to tissue stress. Tissue injury may result from physical increased movement of leukocytes from the blood to the surrounding tissues, increased levels of cytokines, or the increased deposition of compounds such as lipids and cellular debris.

Several lines of evidence indicate that the complement system is central to the pathogenesis of AMD. These include C5 and C5b-9 immunohistochemistry of human drusen and choroidal neovascular (CNV) membranes in a rodent model of exudative AMD; reduced CNV severity in C3-/-, C3AR-/-, and C5AR-deficient mice compared with wild-type littermates; evidence that C3a and C5a trigger increased secretion of vascular endothelial growth factor (VEGF) by human retinal pigment epithelial cells; and compelling evidence that genetic variants in complement genes are major risk factors for the development of AMD. One common variant in the CHI gene is associated with increased risk of twofold to fourfold in heterozygous patients and fivefold to sevenfold in homozygotes. Moreover, variants in complement genes C2/CBF, C3, and SERPING1 have been reported in AMD patients.

Interestingly, although the RPE is generally considered the principal cell type involved in AMD, complement deposits are primarily observed in and around the choriocapillaris, with relatively little localization to the RPE (Fig. 1). In light of the physical association of complement deposits and vascular cells of the choroid, we hypothesized that complement com...
Components might act directly on choroidal endothelial cells through angiogenic or inflammatory mechanisms, or both. In the present study, we sought to determine the effects of complement fragments on the human choriocapillaris and to evaluate how these molecules might promote endothelial cell activation in AMD. Our results suggest that complement complexes that form in the macula may result in elevated macrophage recruitment by increasing endothelial expression of intercellular adhesion molecule-1 (ICAM-1). This could serve as an inflammatory trigger that contributes to the progression of AMD.

METHODS

Human Choroid/RPE Tissue Isolation

All experiments conformed to the Declaration of Helsinki. Human donor eyes were received through the Iowa Lions Eye Bank (Iowa City, IA). Anterior portions were removed for other studies, and the posterior poles were dissected into four leaflets.

For molecular studies of anaphylatoxin receptor mRNA and protein, 6-mm punches of RPE choroid were collected and frozen within 5 hours of death.

For morphologic studies, tissue sections of 15 human donor eyes, collected within 8 hours of death, were prepared that spanned from the macula to the ora serrata. Tissue wedges were fixed in 4% paraformaldehyde in PBS for 2 hours before washing in PBS and embedding, as previously described.

For organ culture experiments, the anterior segment was removed under sterile conditions, and the neuro retina was removed. Retinal pigment epithelial cells were either gently scraped from the Bruch’s membrane/choroid (two eyes) or left intact (two eyes). A 4-mm biopsy punch was used to collect 16 samples, four from each quadrant of the choroid in paired sets.

C3a and C5a Receptor RT-PCR in Human Choroid and Retina

RNA was isolated from frozen punches of human RPE-choroid and human leukocytes using a purification kit according to the manufacturer’s instructions (RNasey Mini Kit; Qiagen, Valencia, CA). Reverse transcription was also performed (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA). PCR was carried out (Biolase DNA Polymerase Kit; Bioline, Taunton, MA) using the following conditions for 35 cycles: denaturing at 94°C for 45 seconds, annealing at 62°C for 30 seconds, and elongation at 72°C for 1 minute. Primers were designed for the amplification of C3a and C5a receptors (C3aR and C5aR): C3aR forward primer, 5'-ACC AGA CAG GAC TCG TGG AG-3'; C3aR reverse primer, 5'-CAC TGG CAA ACA TGT TGA G-3'; C5aR forward primer, 5'-ACC TTC GAT CCT CGG GGA GC-3'; and C5aR reverse primer, 5'-GGG TAC ATG TTG AGC AGG AT-3' (Integrated DNA Technologies, Coralville, IA). Amplified PCR products were separated on a 2% agarose gel (FisherBiotech horizontal electrophoresis system; Fisher Scientific, Waltham, MA) and were stained using ethidium bromide.

C3a and C5a Receptor Detection with Immunoblotting

Retinal and RPE/choroid punches from three human donors were homogenized in PBS with 1% Triton X-100 and complete protease inhibitor (Roche, Indianapolis, IN). Twenty-five micrograms of protein were mixed with an equal volume of 2× Laemmlu buffer, boiled for 5 minutes, separated electrophoretically, and blotted as previously described. Blots were probed with antibodies directed against C3aR and C5aR (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 0.4 μg/mL, followed by washing of the membrane, incubation with 0.4 μg/mL peroxidase-conjugated species-specific secondary antibody (Santa Cruz Biotechnology), and detection using enhanced chemiluminescence (ECL Plus Kit; Amersham Biosciences, Buckinghamshire, UK).

Immunohistochemistry of Complement Proteins and Complement Receptors

Sections of posterior poles spanning the macula to the ora serrata containing both retina and RPE/choroid were collected at a thickness of 7 μm using a cryostat (HM 505 E; Microm International, Walldorf, Germany). Sections were blocked in 1% horse serum for 15 minutes. The primary antibodies used were an antibody directed against C3aR raised in goat at a concentration of 4 μg/mL (Santa Cruz Biotechnology); an antibody directed against C5aR raised in rabbit (Santa Cruz Biotechnology) at 4 μg/mL; and an additional antibody directed against C5aR raised in mouse (AbD Serotec, Raleigh, NC) at 5 μg/mL. Immunolabeling was performed as previously described. In addition, sections of human maculae were dual labeled with antibodies directed against terminal complement complex (15 μg/mL; clone aE11; DAKO, Carpinteria, CA), and Ulex europaeus agglutinin-I using methods previously described.

Evaluation of Choroidal Endothelial Cell Migration and Proliferation in Response to C5a

Migration and proliferation assays were performed on two human primary choroidal endothelial cell lines and the commercially available monkey chorioretinal cell line Rf/6a (ATCC, Manassas, VA). Isolation and purification of primary human cell lines was performed as previously described. For the migration assays, cells...
were seeded into the upper chamber of an 8-μm pore culture insert (Millipore, Billerica, MA). Recombinant human protein was added to the medium on the other side of the insert in a Boyden chamber assay.\textsuperscript{57} Cells were allowed to migrate for 8 hours and were then counted using scanning electron microscopy. For the proliferation assays, equal concentrations of cells were plated and allowed to grow for 48 hours in medium containing different recombinant proteins or BSA. Cells were counted using a flow cytometer (BD LSR II; BD Biosciences, Franklin Lakes, NJ) at the University of Iowa Flow Cytometry Facility. Recombinant human C5a protein was used at a concentration of 0.1 μg/ml for all assays. An equal volume of 2.5 mg/ml BSA was used as a control since this was the C5a protein diluent. All assays were performed in triplicate.

**C5a-Treated Human Choroid Organ Cultures**

Choroidal organ cultures offer the advantage that the intraocular milieu, extracellular matrix, and cell contacts are relatively preserved during the course of an experiment. Choroid punches were collected from four human donors. One punch from four quadrants of the human eye were incubated in 1 μg/ml recombinant C5a protein diluted in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY) with 10% FBS and 1% penicillin/streptomycin. Adjacent punches were incubated in equal volumes of 2.5 mg/ml BSA diluted in the same medium. Incubation was performed for 24 hours at 37°C in 5% CO2. The duration of incubation was well demonstrated positive labeling of bands corresponding to the molecular weight of C5aR protein (approximately 45 kDa).

**Quantification of ICAM-1 Expression in C5a-Treated Human Organ Cultures**

RNA was isolated from four C5a-treated and four control organ culture punches from one human donor (RNeasy Mini Kit; Qiagen). Reverse transcription was then performed (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA), after which quantitative PCR was performed with a sequence detection system (ABI PRISM 7700 with the SYBR Green PCR Master Mix; Applied Biosystems, Foster City, CA), after which quantification was then performed (High-Capacity cDNA Reverse Transcriptase was omitted (--) as a negative control. Reverse transcription was performed as previously described using 2.5 μg/ml anti-ICAM-1 antibody raised in mouse (clone P244; Developmental Studies Hybrida Bank at the University of Iowa).\textsuperscript{54} All sections were developed for 2.5 minutes, a time previously established as within the linear range of development using the same antibody,\textsuperscript{54} rinsed in distilled water, and dehydrated in alcohol and a xylene substitute (Clear-Rite 3; Richard-Allan Scientific, Kalamazoo, MI). Slides were coverslipped (Permount; Fisher Scientific, Pittsburgh, PA), and images were collected for digital imaging (BX-41 microscope [Olympus, Tokyo, Japan] and SPOT-RT camera [Diagnostic Instruments, Detroit, MI]). ICAM-1 morphometry was then performed as previously described.\textsuperscript{54} Briefly, 40 capillaries on average were traced from each punch using the polygon tool in ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Measurements were made from four sections from each individual punch to account for possible variation in section thickness. Average pixel intensity values on a scale of 0 to 255 were collected for each individual capillary in which a higher numerical value indicated a greater labeling intensity of anti-ICAM-1. All measurements were corrected for background by subtracting the average background intensity from the capillary intensity values per image. Comparisons were analyzed using a two-way paired Student’s t-test.

**C5R1 and C3AR1 Genotyping**

The study was approved by the University of Iowa’s institutional review board, and informed consent was obtained from study participants. A cohort of 329 subjects with AMD and 322 control subjects from Iowa were enrolled using standard criteria.\textsuperscript{40} The cohorts were genotyped at five single-nucleotide polymorphisms (SNPs) within the C3AR1 gene and two SNPs within the C5R1 gene (rs116790330 and rs4427917) using a mass spectroscopy-based system (Sequenom, San Diego, CA). Genotyping was conducted using the MassArray platform and reagents according to the manufacturer’s protocol (iPlex Gold; GeneSeek, Lincoln, NE). SNP genotypes were compared between AMD patients and controls using chi-square analysis, and allele frequencies were compared using Fisher’s exact test. Bonferroni correction was used to adjust P values for multiple measures as needed.

**Results**

**C3a and C5a Receptor RT-PCR in Human Choroid and Retina**

To analyze the presence of C3aR, C5aR, or both, in the retina/choroid complex, PCR was performed on cDNA from human

![Image](https://example.com/figure_2.png)

**Figure 2.** Expression of anaphylatoxin receptors in human eyes (A) C5aR and C5AR RT-PCR of human RPE/choroid (Ch). Human leukocytes (WBC) were used as a positive control. C3aR and C5aR transcripts were both found in leukocytes. C5aR, but not C3aR, was expressed in the RPE/choroid. (B) C5aR RT-PCR results using retina and RPE/choroid cDNA (n = 5). Retina and RPE/choroid express C5aR in all tested donors. Reverse
retina tissue and human RPE/choroid tissue (n = 3 human donors). C3aR and C5aR, both expressed by human leukocytes, were used as a positive control (Fig. 2A). Transcripts for C5aR were found in both the retina and the choroid (Fig. 2B). In contrast, C3aR transcripts were not found in the retina or choroid (Fig. 2A).

C3a and C5a Receptor Identification Using Immunoblotting

The expression of C5aR protein was detected in both the retina and the RPE/choroid tissues separately (n = 3; a representative example of this result is depicted in Fig. 2B). A single major band was observed at approximately 40.4 kDa (Fig. 2C). A less abundant, higher molecular weight band, possibly indicative of C5aR dimers, was observed in both tissues. C3aR was not detected for any of the tissues evaluated (data not shown).

C3a and C5a Receptor Localization Using Immunohistochemistry

To determine the cell types in which C3a and C5a may be capable of eliciting physiological responses in the RPE/choroid complex, immunohistochemistry was performed using anti-C3aR and anti-C5aR antibodies on sagittal sections of human donor eye tissues. We found that anti-C5aR antibodies localized to most vessels in the choroid, including the choriocapillaris, and to the basal surface of the RPE (n = 15). The same vascular labeling pattern was found using two different antibodies (Figs. 3A, 3B). Sections incubated with anti-C3aR antibody did not exhibit any retinal or choroidal labeling (Fig. 3C).

Evaluation of Choroidal Endothelial Cell Migration and Proliferation in Response to C5a

To determine whether C5a had any proangiogenic effects on the endothelial cells of the choroid, migration and proliferation assays with human and monkey endothelial cells were used as previously described. There were no significant changes in migration or proliferation for either cell type in the presence of recombinant C5a protein compared with controls (data not shown), suggesting that C5a does not promote angiogenesis in choroidal endothelial cells.

Quantification of ICAM-1 Expression in C5a-Treated Human Organ Cultures

Quantitative RT-PCR of ICAM1 cDNA showed a 13.1% increase in ICAM-1 expression in tissues treated with C5a in comparison with the BSA-treated controls at 16 hours (P = 0.02; data not shown). ICAM-1 protein levels, normalized to β-actin levels,
Results are divided into two groups, choroid with RPE and choroid without RPE, indicating the two methods used to prepare the human donor tissue punches. Average ICAM-1 intensity values are given on a scale from 0 to 255, where 255 indicates the highest possible level of ICAM-1 intensity (i.e., saturation). For both methods, tissues exposed to C5a protein exhibited higher average ICAM-1 intensities than control tissues.

**ICAM-1 Morphometry of C5a-Treated Human Choroid Organ Cultures**

Histologic samples of organ cultures were evaluated to determine the site of C5a-dependent ICAM-1 expression. Endothelial cells of the choriocapillaris showed increased labeling after C5a treatment (Figs. 4B, 4C). Organ culture punches (C5a treated and untreated) from samples of eyes with the RPE (n = 2 donors) and without the RPE (n = 2 donors) were analyzed separately to determine whether having another cell type (retinal pigment epithelial) present during C5a incubation affects the expression of ICAM-1. After morphometric analysis, it was found that both groups of punches exhibited an increased ICAM-1 labeling intensity on endothelial cells exposed to C5a protein compared with controls. There was a 13.9% higher average intensity for endothelial cells in punches with RPE and a 9.5% higher average intensity for endothelial cells in punches without RPE. For both groups, P < 0.001 (Table 1).

**Association Study of C3AR1 and C5R1 in AMD**

A cohort of 329 AMD patients and 322 controls was genotyped at SNPs within the C3AR1 gene (five SNPs) and the C5R1 gene (two SNPs) using mass spectroscopy. No significant difference was detected between the allele frequencies and genotypes of SNPs in either the C3AR1 or the C5R1 gene (P > 0.05; Table 2).

**Discussion**

In this study, we have shown that the gene encoding the C5a receptor is expressed in the human choroid and retina in vivo by PCR. We have further shown that within the choroid, C5aR is expressed in the endothelium of the choriocapillaris. We did not find convincing expression or localization of the receptor for C3a in the choroid or the retina using PCR or immunohistochemistry. These results suggest that complement cascade component C5a is more likely to be responsible for activating endothelial cells than C3a. We therefore focused on C5a for subsequent functional experiments.

No significant changes were observed in the migratory or proliferative responses of choroidal endothelial cells to C5a protein. These results suggest that the effect of C5a on endothelial cell activation is not through promoting angiogenesis, although it is possible that cell culture conditions do not replicate the effects of C5a in vivo. After further investigation, we found that the activation of human choroidal endothelial cells was more closely linked to inflammatory mechanisms of cell activation.

Evaluation of ICAM-1 expression in the choroid after C5a exposure, using quantitative PCR, Western blot analysis, and ICAM-1 morphometry, showed that human choroid punches exposed to C5a, in contrast to those exposed to BSA alone, increased the expression and localization of ICAM-1 in the choriocapillaris. This result was not dependent on the presence of the RPE, suggesting that C5a may act directly on choroidal endothelial cells. Although the overall increase in ICAM-1 expression was modest, it was comparable to the macular-peripheral differences observed previously. Even a small chronic increase in adhesion molecule expression might result in increased monocyte interaction, especially over the decades during which AMD develops.

ICAM-1 plays a role in the infiltration of leukocytes into surrounding tissues during inflammation through its interaction with CD11/CD18 integrins on circulating leukocytes. An increased level of ICAM-1 on the choroidal endothelium likely enhances leukocyte trafficking from the blood as well. Mice deficient for C5aR or ICAM-1 both demonstrate a significant reduction in neovascular membrane volume after laser induction. Given that the present study demonstrates that C5a increases choroidal endothelial expression of ICAM-1, it is feasible that the lack of ICAM-1-mediated leukocyte recruitment in these knockout mice is responsible for the reduction in neovascular membrane severity. The role of different classes of leukocytes in AMD pathogenesis is controversial. One possible outcome of increased leukocyte recruitment is that capillary endothelial cells may become damaged by the adhesion of neutrophils or monocytes. Moreover, after extravasation, macrophages can potentially promote CNV through a variety of processes. Complement-mediated activation of choroidal endothelial cells could advance disease in AMD through an ischemic/atrophic mechanism or through angiogenic events.

Finally, we explored the role of genetic variants in the genes for C3AR and C5AR (C5R1 and C3AR1) in the pathogenesis of AMD by conducting a focused association study. However, we

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**Table 1. ICAM-1 Morphometry Results**

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<th>Sample</th>
<th>Condition</th>
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<th>SEM</th>
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<tr>
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<td>148.1</td>
<td>1.5</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
<td>BSA</td>
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**Table 2. Results of SNP Screening in AMD and Control Samples**

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<tr>
<th>Gene</th>
<th>SNP</th>
<th>Samples Genotyped (%)</th>
<th>AMD Major/Minor Allele</th>
<th>Control Major/Minor Allele</th>
<th>P (uncorrected)</th>
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<td>.70/.30</td>
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</table>
did not observe any association between SNPs in the C3AR1 and C5R1 genes and AMD. These data suggest that ancestral variants in these genes are not common risk factors for AMD.

There were a number of limitations to this study. First, in our in vitro studies, we were unable to fully model the complex interactions that undoubtedly occur in aging eyes with AMD. The pathogenesis of AMD is likely to be complex, with interactions between retinal pigment epithelial, endothelial, and myeloid cells, each of which may be capable of responding to complement molecules. Studies in organ culture, where at least some of the functional and structural interactions are preserved in vitro, partially mitigate these challenges. Results from these studies suggest that the chorio-capillaris can respond to C5a directly, with or without interaction with the RPE. In addition, our results showing that C5a is not sufficient to induce migration and proliferation in choroidal endothelial cells does not prove that C5a does not affect this behavior indirectly through the RPE, as suggested previously. It does, however, suggest that C5a does not act directly on choroidal endothelial cells to induce angiogenic behaviors, in contrast to VEGF and elastin fragments.

In summary, we observed C5αR, but not C3αR, in the human chorio-capillaris in vivo and found that C5α activates choroidal endothelial cells to increase ICAM-1 expression at the protein and mRNA levels. This increased expression is at the level of the chorio-capillaris. The presence of monocyte-derived cells in AMD eyes has been noted (see Ref. 47 for review), as has a key role for the complement system. The complement anaphylatoxin receptors and membrane-bound regulators may provide an attractive target for novel therapies for AMD. These data suggest that ancestral variants in these genes are not common risk factors for AMD.

Acknowledgments
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References