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Monomeric C-reactive protein and inflammation in age-related macular degeneration

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Abstract

Age-related macular degeneration (AMD) is a devastating disease characterized by central vision loss in elderly individuals. Previous studies have suggested a link between elevated levels of total C-reactive protein (CRP) in the choroid, CFH genotype, and AMD status; however, the structural form of CRP present in the choroid, its relationship to CFH genotype, and its functional consequences have not been assessed. In this report, we studied genotyped human donor eyes ($n = 60$) and found that eyes homozygous for the high-risk CFH (Y402H) allele had elevated monomeric CRP (mCRP) within the choriocapillaris and Bruch's membrane, compared to those with the low-risk genotype. Treatment of choroidal endothelial cells in vitro with mCRP increased migration rate and monolayer permeability compared to treatment with pentameric CRP (pCRP) or medium alone. Organ cultures treated with mCRP exhibited dramatically altered expression of inflammatory genes as assessed by RNA sequencing, including ICAM-1 and CA4, both of which were confirmed at the protein level. Our data indicate that mCRP is the more abundant form of CRP in human choroid, and that mCRP levels are elevated in individuals with the high-risk CFH genotype. Moreover, pro-inflammatory mCRP significantly affects endothelial cell phenotypes in vitro and ex vivo, suggesting a role for mCRP in choroidal vascular dysfunction in AMD.

Keywords

C-reactive protein; mCRP; age-related macular degeneration; choriocapillaris; choroidal endothelial cells; CFH; angiogenesis

INTRODUCTION

As a leading cause of irreversible vision loss in developed countries [1], age-related macular degeneration (AMD) is a devastating disease affecting millions of people in the United States alone. The term AMD encompasses at least three primary clinical forms of disease, including early dry (non-exudative) AMD, late dry (non-exudative) AMD, and wet (exudative) AMD. This disease is often recognized ophthalmoscopically by the presence of drusen, retinal pigment epithelium (RPE) changes, regions of atrophy, and/or choroidal

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RNA-Seq data are appended as Supplemental Table 2.

neovascular membranes within the central part of the retina known as the macula [2, 3]. Patients with wet AMD typically benefit from intravitreal anti-vascular endothelial growth factor (VEGF) injections to arrest the growth of choroidal neovascular membranes and facilitate the removal of fluid from the retina; however, any cell loss that occurs with advanced AMD currently cannot be treated. AMD has a complex aetiology that involves the loss of photoreceptor cells, RPE, and choroidal endothelial cells within the macula. The exact cause or order of these events is currently not fully understood; however, much progress has been made in the discovery of risk factors for the disease.

Genetics play an important role in AMD risk, with at least 34 different loci having a significant association with AMD [4]. One of the most significantly associated genetic risk factors is a single nucleotide polymorphism (SNP) in the CFH gene that results in a tyrosine-to-histidine substitution at amino acid 402 (Y402H) of the complement factor H (CFH) protein. The discovery that this CFH variant confers an increased risk for AMD development provided a link between the disease and complement activation [5–8].

Another inflammatory risk factor associated with AMD is elevated serum C-reactive protein (CRP), which has long been studied as a marker of systemic inflammation. As a member of the pentraxin protein family, which includes AMD-associated PTX3 [9], CRP is an acute phase protein predominantly produced in the liver and released into circulation upon stimulation by IL-6 and other cytokines [10]. CRP primarily exists in two structurally and functionally independent forms: (1) net anti-inflammatory, serum-associated pentameric CRP (pCRP), and (2) pro-inflammatory tissue-associated, monomeric CRP (mCRP). Within 8–72 h of the onset of an inflammatory response, serum pCRP concentrations have been shown to increase from less than 3 µg/ml to more than 100 µg/ml [11–13]. The circulating 115 kDa pCRP homopentamer irreversibly dissociates into 23 kDa monomers upon binding to pathogenic membranes, damaged or apoptotic cell membranes, activated platelets, or blood-derived microparticles [14–17]. mCRP is mainly tissue-associated, as its formation requires a conformational change that renders the protein largely insoluble [18, 19].

While there is currently no evidence for a significant association between common CRP SNPs and risk of AMD development [20], elevated serum CRP levels have been linked to disease progression [21–23], with an additive effect resulting from the presence of high levels of both CRP and the high-risk CFH allele [24]. Seddon et al found that serum CRP concentrations at < 0.5, 0.5–10.0, and >10.0 µg/ml correspond to a low risk, a normal risk, and a high risk of AMD progression, respectively [22].

Previous work has focused on an association between total CRP levels, CFH genotype, and AMD; however, the relationship between CFH genotype and each of the two functionally opposed forms of CRP has not been previously evaluated. In addition, while mCRP binding to necrotic RPE cells has been observed [16], the functional ramifications of elevated mCRP on cells affected by AMD have yet to be assessed. In light of the previously observed associations between CRP and AMD, a better understanding of the role that CRP plays in AMD pathogenesis and progression is required. Here, we sought to examine the presence and relative abundance of the two main forms of CRP, pCRP and mCRP, in the human choroid in a large cohort of genotyped donor eyes based on CFH genotype. In addition, we

studied the effects of mCRP and pCRP proteins on endothelial cell function in vitro, and performed RNA sequencing (RNA-Seq) analysis on mCRP-exposed human choroid ex vivo to identify the direct effects of elevated mCRP on choroidal endothelial cell gene expression in human eyes.

MATERIALS AND METHODS

Tissue collection

Donated human eyes were obtained from the Iowa Lions Eye Bank (Iowa City, IA, USA), following informed consent from the donors' families, and were preserved within 8 h of death. All experiments adhered to the Declaration of Helsinki. One eye from each of 60 donors was employed for histological experiments; one eye from each of 14 donors was used for native-PAGE western blotting and ELISA experiments; and one eye from each of four donors was utilized for organ culture experiments.

Genotyping—Whole blood or extraocular muscle tissue was collected from each donor, and DNA was isolated using the DNeasy kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Genotyping at the rs1061170 SNP was performed using TaqMan genotyping assays (Applied Biosystems, Foster City, CA, USA) in a high-throughput microfluidics system (Fluidigm, San Francisco, CA, USA) [25].

Recombinant mCRP

Recombinant human mCRP (rmCRP) was produced as described previously [26]. The protein was mutated to replace both cysteine residues with alanine residues (i.e. C36A and C97A) and was further modified using a reverse acylation procedure to enhance aqueous solubility [26, 27]. Based on testing using the ToxinSensor™ Gel Clot Endotoxin Assay Kit (GenScript; cat# L00351; Piscataway, NJ, USA), 20 µg/ml mCRP contains less than 0.125 EU/ml (~0.025 ng/ml) endotoxin levels. Endotoxin test details can be found in the Supplementary materials and methods. The rmCRP recombinant protein will be referred to as 'mCRP' for all experiments discussed herein.

Immunohistochemistry—An 8-mm diameter punch of full-thickness retina (neural retina, RPE, choroid, and sclera) tissue centred on the fovea centralis, the very centre of the macula, and a 6-mm diameter punch from the peripheral retina were collected from each eye. The punches were fixed in 4% paraformaldehyde and cryoprotected by passing through a sucrose gradient before being embedded in 20% sucrose in Optimal Cutting Temperature compound (VWR, Radnor, PA, USA). Seven-micrometre-thick sections were collected from each sample.

For colorimetric detection, primary monoclonal antibodies directed against pCRP (1D6) or mCRP (3H12) were utilized. These monoclonal antibodies have previously been shown to be highly specific for their respective targets [26]. Detailed immunohistochemistry methods are available in the Supplementary materials and methods.

Semi-native PAGE western blotting—Total protein was extracted from 6-mm RPE/choroid punches obtained from four donors homozygous for the CFH Y allele, five donors

homozygous for the H allele, and five heterozygous donors, and concentrations were determined using the BioRad DC Assay according to the manufacturer's instructions (BioRad, Hercules, CA, USA). For each sample, 20 µg of protein was diluted in PBS with 2 mM CaCl₂ and 140 mM NaCl mixed with Native Sample Buffer (BioRad) and loaded into a 10% Mini-PROTEAN TGX precast gel (BioRad). Semi-native PAGE separation was performed using 0.005% SDS. Proteins were then blotted onto a polyvinylidene difluoride (PVDF) membrane (BioRad). Membranes were treated briefly with methanol followed by blocking with 1% BSA (Research Products International, Mt Prospect, IL, USA) in PBS with 0.1% Tween-20 (PBS-T; Research Products International). To detect both forms of CRP, the membrane was incubated with primary antibodies directed against pCRP (1D6) or mCRP (3H12) for 2.5 h. Following three 5-min washes in PBS-T, membranes were treated with Alexa Fluor® 488-conjugated donkey anti-mouse secondary antibody (A-21202; Thermo Fisher Scientific, Waltham, MA, USA) and washed three times for 5 min each in TBS with 0.1% Tween-20 (TBS-T; BioRad). Finally, bands were detected using a VersaDoc Imager (BioRad).

ELISA—RPE/choroid protein extraction was performed, using the same 15 donor tissues used for the semi-native PAGE western blotting assay, and protein concentrations were determined as described above. For each sample, 128 µg of total protein was loaded in duplicate into the appropriate wells of a commercial human CRP ELISA plate (ELH-CRP; RayBiotech, Norcross, GA, USA). The assay was performed according to the manufacturer's instructions. Optical density was measured at 405 nm using an EZ Read 400 Microplate Reader (Biochrom, Holliston, MA, USA).

Scratch closure assay—Monkey choroidal endothelial (RF/6A) cells (ATCC, Manassas, VA, USA) were plated on a 24-well plate (Costar, Cambridge, MA, USA) and grown in media consisting of Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA), 5% fetal bovine serum (FBS; ATCC), and 1% penicillin streptomycin (Life Technologies) until they formed a confluent monolayer. Using a pipette tip, a line was drawn down the centre of each monolayer to form a scratch devoid of cells. The media were removed from each well and replaced with 500 µl of one of the following: fresh media alone (n = 4 wells per assay), 20 µg/ml pCRP (AG723; EMD Millipore, Temecula, CA, USA) in media (n = 8 wells per assay), or 20 µg/ml recombinant mCRP in media (n = 8 wells per assay). Using an Olympus IX-81 inverted microscope with a 10× lens and a DP71 camera, a bright-field image was taken of each scratch at the time of scratch formation (0 h) and every 12 h after until the first scratch was closed. The percentage of scratch closure was calculated using the following equation: $100 - [(\text{gap width at time X} / \text{initial gap width}) \times 100]$. Experiments were repeated three times and average values were used for statistical analyses.

Transendothelial resistance (TER) assay—RF/6A cells were plated and grown on the apical side of a Transwell insert until confluent. The media in the insert were replaced with one of the following solutions: fresh media only (n = 3 wells per assay), 20 µg/ml pCRP in media (n = 3 wells per assay), or 20 µg/ml mCRP in media (n = 3 wells per assay). Three wells containing media only, without any cells, were used as the baseline TER measurements. All solutions were made using DMEM, 5% FBS, and 1% penicillin

streptomycin. The TER across the monolayer was measured using EVOM2 chopstick electrodes (STX2; World Precision Instruments, Sarasota, FL, USA) at the following time points after the solution was added to the cells: 0 min (when solution was added), 15 min, 30 min, 2 h, and 4 h. Experiments were repeated three times and average values were used for statistical analyses.

Organ culture—Six-millimetre punches were obtained from the peripheral retina of healthy donor eyes. Neural retina and sclera were removed, and punches consisting of the RPE and choroid were immediately cultured for 12 h (n = 6 punches for each of three replicates) or 24 h (n = 8 punches) at 37°C in media only (DMEM, 5% FBS, and 1% penicillin streptomycin) or media supplemented with 20 µg/ml mCRP. At the end of the incubation period, all tissue samples were flash-frozen in liquid nitrogen for biochemical or RNA-Seq analysis.

RNA preparation, sequencing, and bioinformatics—Total RNA was extracted from six RPE/choroid organ culture punches using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. In brief, tissue was homogenized in Buffer RLT and centrifuged for 3 min at maximum speed on a bench centrifuge. An equal volume of 70% ethanol was added to each supernatant, mixed, transferred to a spin column, and centrifuged for 15 s at 8000 × g. On-column DNase digestion was carried out by adding Buffer RW1 to the column, centrifuging for 15 s at 8000 × g, treating with DNase I for 15 min, and then adding Buffer RW1 and centrifuging again. Buffer RPE was then added to the column, which was subsequently centrifuged at 8000 × g for 2 min. To elute the RNA, RNase-free water was added directly to each column and the flow-through was collected. RNA concentrations were measured using a NanoDrop spectrophotometer (Thermo Scientific, Rockford, IL, USA), and the remaining RNA samples were stored at −80°C.

RNA-Seq libraries were prepared using the KAPA Stranded mRNA-Seq Prep Kit (Kapa Biosystems, Wilmington, MA, USA) and sequenced as 100-nucleotide paired-end reads on an Illumina HiSeq platform (Illumina, Madison, WI, USA) by the Genomics Division of the Iowa Institute for Human Genetics. Additional bioinformatics information may be found in the Supplementary materials and methods.

Western blotting—The RPE/choroid was collected from each punch after organ culture and homogenized in the Complete protease inhibitor kit (Roche Diagnostic, Indianapolis, IN, USA) in PBS with 1% Triton X-100 for protein extraction. Total protein concentrations were determined as described above. For each sample, 20 µg of protein was mixed with Laemmli buffer plus β-mercaptoethanol and loaded into a 4–20% Mini-PROTEAN TGX precast gel (BioRad) for SDS-PAGE separation. Blotting and antibody incubations steps were then carried out as described above and primary antibodies directed against intercellular adhesion molecule-1 (ICAM-1; BBA17; R&D Systems, Minneapolis, MN, USA) and carbonic anhydrase IV (CA4; MAB21861; R&D Systems) were used.

Statistical analysis—RF/6A scratch closure and TER assays, as well as mCRP, pCRP, ICAM-1, and CA4 protein level analyses, were assessed using a two-tailed Student's t-test.

A Fisher's exact test was utilized to assess IHC data. Because all assays were repeated at least three times, data points with an uncorrected p-value < 0.05 were considered significant.

RESULTS

mCRP is elevated in the choriocapillaris and Bruch's membrane of individuals with the high-risk CFH genotype

In order to individually assess the abundance of both mCRP and pCRP, immunohistochemistry was performed using anti-pCRP and anti-mCRP antibodies on genotyped human choroid tissue. With increased immunolabelling compared with pCRP, we found mCRP to be the main form of the protein present in the choriocapillaris and Bruch's membrane (Figure 1A–F). In addition, masked scoring was performed for each section, and eyes homozygous for the high-risk CFH allele were found to have elevated mCRP immunolabelling compared with low-risk eyes ($p < 0.05$; Figure 1G). Table S1 (supplementary material) contains a summary of the immunolabelling results for all 50 samples. An example of the scoring system can be found in Figure 1H.

ELISA using protein extracted from genotyped RPE/choroid showed highly variable levels of total CRP, with a trend towards increased abundance in eyes with one or more copies of the CFH risk allele (Figure 2A). Similarly, semi-native western blotting analyses with human RPE/choroid tissue revealed variable abundance of pCRP (115 kDa), regardless of genotype, but showed a significant ($p < 0.05$) increase in the abundance of mCRP (23 kDa) in choroids that harboured at least one copy of the CFH variant (Figure 2B–D).

mCRP is present in sub-RPE deposits associated with AMD

Sub-RPE drusen formation within the macula is associated with an increased risk of AMD development and progression [28]. Understanding the components of these extracellular deposits could help to provide insight into the molecular events involved in their formation. Since CRP has previously been evaluated and is a confirmed component of drusen [29], immunohistochemistry was employed to identify the forms of CRP present in various types of drusen. mCRP was observed in the highly homogeneous small drusen (Figure 3A) and in heterogeneous large soft drusen (Figure 3E), but not in large, hyaline drusen (Figure 3C). pCRP was absent from all sub-RPE deposits (data not shown).

Choroidal endothelial cell migration is significantly increased by mCRP treatment

Due to its localization around the choriocapillaris, and its association with neovascular AMD [30], we assessed the effect of mCRP on choroidal endothelial cell migration. We found that mCRP accelerated the rate of scratch closure for cultured RF/6A cells at 36 h ($p < 0.01$), 48 h ($p < 0.005$), and 60 h ($p < 0.005$) after treatment. Interestingly, there was also a significant increase in the scratch closure rate for pCRP treatment at 48 h ($p < 0.05$) and 60 h ($p < 0.01$) after treatment (Figure 4A), which is likely due to spontaneous dissociation from pCRP to mCRP, a process that begins to occur in as little as 4 h post-treatment [19, 31–33].

Treatment with mCRP rapidly decreases the resistance across choroidal endothelial cell monolayers

One early step in pathological angiogenesis is an increase in transendothelial permeability [34]. Therefore, we investigated the extent to which mCRP increases vascular permeability using a transendothelial resistance (TER) assay. Compared with control cells, mCRP-treated cells showed significantly reduced TER at 30 min ($p < 0.005$) and 120 min ($p < 0.05$) post-treatment (Figure 4B), indicating a rapid mCRP-mediated increase in choroidal endothelial cell monolayer permeability.

Ex vivo treatment with mCRP stimulates pro-inflammatory gene expression in RPE/choroid tissue—To gain an understanding of how the transcriptome changes within the RPE/choroid after 12 h exposure to mCRP, RNA sequencing was performed on human tissue ex vivo. All RNA-Seq results are provided as Table S3 (supplementary material). A total of 14 315 genes were expressed above 10 reads in all replicates of either the mCRP or the control group, and a total of 43 genes were identified as differentially expressed by DESeq2 and Cuffdiff (Figure 5; supplementary material, Table S2). The top 29 most significantly up-regulated genes were submitted to the WebGestalt database (<http://bioinfo.vanderbilt.edu/webgestalt/>) to perform gene ontology enrichment analysis. The most significantly altered biological processes are shown in Table 1.

ICAM-1 gene expression was significantly increased (DESeq2 adjusted p -value $< 2.79\text{E-}13$, $\log_2$ fold change = 1.1; Cuffdiff adjusted p -value < 0.002 , $\log_2$ fold change = 1.4), while CA4 expression was down-regulated (DESeq2 adjusted p -value $< 1.41\text{E-}09$, $\log_2$ fold change = -1.32 ; Cuffdiff adjusted p -value < 0.002 , $\log_2$ fold change = -1.91) in RPE/choroid tissue treated with mCRP compared with untreated tissue. A complete list of the most significantly up-regulated and down-regulated genes with a $\log_2$ fold change ≥ 1 or ≤ -1 , respectively, after mCRP treatment can be found in Table S2 (supplementary material).

mCRP increases ICAM-1 and decreases CA4 levels in the human choroid—Changes in mRNA levels may not perfectly reflect changes in protein abundance; therefore, western blotting was performed on our ex vivo RPE/choroid tissue to determine if protein levels corroborate the RNA-Seq data. ICAM-1 protein levels were significantly increased ($p < 0.05$) in human RPE/choroid tissue after the 12-h mCRP treatment compared with control, which parallels the RNA-Seq results (Figure 6A). Surprisingly, ICAM-1 protein levels were not significantly altered at the 24-h time point (Figure 6B). In contrast to the short-term decrease in CA4 mRNA expression in RNA-Seq experiments, CA4 protein levels remained unchanged after 12 h of mCRP treatment (Figure 6C). However, after 24 h, CA4 levels were significantly decreased ($p < 0.05$) in the mCRP-treated RPE/choroid tissue compared with those of untreated tissue (Figure 6D).

DISCUSSION

Johnson et al assessed the localization and relative abundance of both CFH and total CRP in the retina of individuals homozygous for the high-risk CFH Y402H allele or those homozygous for the normal CFH allele. Unlike CFH, total CRP immunolabelling was significantly higher in the choroid of at-risk individuals, regardless of disease status [35]. An

inverse relationship between CFH and total CRP immunolabelling in the choroid of AMD-afflicted eyes has also been reported [30]. Compared with healthy aged controls, total CRP was found to be elevated in early and wet AMD eyes, while CFH was lower in AMD eyes, regardless of subtype or severity [30].

In the current study, we evaluated CRP isoform distribution and the functional consequences of increased mCRP on endothelial cell dysfunction in age-related macular degeneration. Here, we have shown that mCRP, but not pCRP, levels are higher in the choroid of individuals with the high-risk CFH genotype and that in vitro treatment with recombinant mCRP leads to increased choroidal endothelial cell migration and monolayer permeability. Finally, we have provided novel data regarding the RPE/choroid transcriptome in response to ex vivo mCRP stimulation, which show an increase in immune response-related gene expression.

These data provide evidence for a link between mCRP abundance in the choriocapillaris and AMD risk. Previous work has also provided strong evidence for how mCRP affects endothelial cell function, by binding to lipid moieties and inserting directly into human aortic endothelial cell membranes after just 1 h of mCRP exposure in vitro. This mCRP–human aortic endothelial cell interaction, and the subsequent activation of the endothelial cells, is largely mediated through lipid raft binding by mCRP [36], which requires reduction of the intrasubunit disulfide bond within the mCRP monomer [37]. In addition to its CFH binding capabilities [16], in vitro studies have shown that recombinant mCRP, but not pCRP, readily binds to immobilized C1q, leading to significant C1 complex activation, which, in turn, initiates the classical pathway of complement activation [38]. Complement activation ultimately leads to the formation of the membrane attack complex (MAC). Levels of the MAC are higher in the choriocapillaris than in other microvascular beds [39], and its abundance has been shown to increase with age [40]. Importantly, donor eyes homozygous for the CFH (Y402H) variant have elevated MAC [25]. In support of its previously reported interaction with C1q, mCRP was observed to co-localize with the MAC (C5b-9) in the choriocapillaris (supplementary material, Figure S1). Therefore, as a pro-inflammatory molecule that is known to interact with endothelial cells and positively regulate complement activation, mCRP likely acts to propagate inflammation in the choriocapillaris. Furthermore, since elevated mCRP is present in many of the examined choroids prior to the onset of AMD, its accumulation may be an early event in the pathogenesis of the disease. Interestingly, a recent study has suggested an association between statin treatment and drusen regression in a small cohort [41], and statins have separately been shown to decrease the levels of serum CRP in coronary artery disease patients [42]. Therefore, it is possible that the mCRP that we observed within drusenoid deposits is important in their growth or formation.

While it has no effect on proliferation, mCRP treatment has previously been shown to significantly increase endothelial cell migration and tube formation, and induce angiogenesis [43]. In line with these data, we observed a significant effect of mCRP treatment on choroidal endothelial cell migration. A rapid increase in endothelial cell monolayer permeability was also detected after mCRP treatment, suggesting a role for mCRP in increasing vascular permeability to pathological exudate or leukocytes.

Whereas most other vascular beds only express ICAM-1 in an immune state, the vessels that make up a healthy choriocapillaris constitutively express this inflammatory molecule [44], especially those within the macula [45]. Here, we have shown that mCRP exposure further elevates ICAM-1 expression in the choroid compared with untreated tissue in as little as 12 h. As elevated ICAM-1 has previously been associated with AMD [46–48], these data suggest an important role for mCRP in disease pathogenesis through stimulating the up-regulation of ICAM-1. An increase in ICAM-1 expression promotes increased leukocyte recruitment into the choroid [49]. If these events become chronic, as suggested in AMD, significant tissue damage is likely to result. Based on the data presented here, mCRP may act to propagate the inflammatory response within the choroid, leading to elevated ICAM-1 and increased leukocyte recruitment.

As a metalloenzyme associated with both luminal and abluminal choriocapillary endothelial cell membranes, carbonic anhydrase IV (CA4) acts to regulate local pH and is thought to assist in the transport of CO₂ and ions into and out of capillaries [50]. Compared with other endothelial cell populations, expression of CA4 is enriched in mature choroidal endothelial cells [51]. Therefore, reduced CA4 gene expression, which is eventually followed by protein loss in these experiments, could indicate endothelial cell dedifferentiation or altered function, which, if mCRP exposure persists, may lead to severe endothelial cell dysfunction or loss altogether. Notably, CA4 protein levels are decreased in the choroid of AMD eyes [52]. One hypothesis for AMD pathogenesis includes the early loss of endothelial cells that make up the choriocapillaris [53–55]. Upon major loss of vasculature, the surrounding tissue can become hypoxic, leading to increased ICAM-1 expression on the remaining endothelial cells [47, 48] as well as elevated levels of VEGF in the choriocapillaris [56, 57]. As a potent stimulator of angiogenesis, VEGF expression above normal levels could cause the aberrant vessel growth commonly observed in wet AMD. Taken together, we hypothesize that accumulation of mCRP in the choriocapillaris initiates or propagates inflammation within the choroid, through its ability to elevate inflammatory proteins, activate complement along the classical pathway, increase endothelial cell dysfunction, induce endothelial cell permeability, and stimulate endothelial cell angiogenesis, all of which contribute to choroidal neovascularization in AMD.

Given the role that mCRP likely plays in AMD, it will be of great importance to develop therapeutic agents to target the effects of mCRP in the choriocapillaris, including complement-mediated inflammation. Notably, agents that suppress immune-mediated processes, including complement activation, are currently being tested or developed as potential therapies for AMD [58–61].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Friedman DS, O'Colmain BJ, Muñoz B, et al. Prevalence of age-related macular degeneration in the United States. *Arch Ophthalmol*. 2004; 122:564–572. [PubMed: 15078675]
2. Bird AC, Bressler NM, Bressler SB, et al. An international classification and grading system for age-related maculopathy and age-related macular degeneration. *Surv Ophthalmol*. 1995; 39:367–374. [PubMed: 7604360]
3. Age-Related Eye Disease Study Research Group. The Age-Related Eye Disease Study (AREDS): design implications. AREDS report no. 1. *Control Clin Trials*. 1999; 20:573–600. [PubMed: 10588299]
4. Fritsche LG, Igl W, Bailey JNC, et al. A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants. *Nature Genet*. 2016; 48:134–143. [PubMed: 26691988]
5. Edwards AO, Ritter R, Abel KJ, et al. Complement factor H polymorphism and age-related macular degeneration. *Science*. 2005; 308:421–424. [PubMed: 15761121]
6. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science*. 2005; 308:419–421. [PubMed: 15761120]
7. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science*. 2005; 308:385–389. [PubMed: 15761122]
8. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci USA*. 2005; 102:7227–7232. [PubMed: 15870199]
9. Juel HB, Faber C, Munthe-Fog L, et al. Systemic and ocular long pentraxin 3 in patients with age-related macular degeneration. *PLoS One*. 2015; 10:e0132800. [PubMed: 26176960]
10. Ganter U, Arcone R, Toniatti C, et al. Dual control of C-reactive protein gene expression by interleukin-1 and interleukin-6. *EMBO J*. 1989; 8:3773. [PubMed: 2555173]
11. Morley JJ, Kushner I. Serum C-reactive protein levels in disease. *Ann NY Acad Sci*. 1982; 389:406–418. [PubMed: 6953917]
12. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med*. 1999; 340:448–454. [PubMed: 9971870]
13. Black S, Kushner I, Samols D. C-reactive protein. *J Biol Chem*. 2004; 279:48487–48490. [PubMed: 15337754]
14. Eisenhardt SU, Habersberger J, Murphy A, et al. Dissociation of pentameric to monomeric C-reactive protein on activated platelets localizes inflammation to atherosclerotic plaques. *Circ Res*. 2009; 105:128–137. [PubMed: 19520972]
15. Molins B, Peña E, la Torre de R, et al. Monomeric C-reactive protein is prothrombotic and dissociates from circulating pentameric C-reactive protein on adhered activated platelets under flow. *Cardiovasc Res*. 2011; 92:328–337. [PubMed: 21859817]
16. Lauer N, Mihlan M, Hartmann A, et al. Complement regulation at necrotic cell lesions is impaired by the age-related macular degeneration-associated factor-H His402 risk variant. *J Immunol*. 2011; 187:4374–4383. [PubMed: 21930971]
17. Habersberger J, Strang F, Scheichl A, et al. Circulating microparticles generate and transport monomeric C-reactive protein in patients with myocardial infarction. *Cardiovasc Res*. 2012; 96:64–72. [PubMed: 22798388]
18. Potempa LA, Maldonado BA, Laurent P, et al. Antigenic, electrophoretic and binding alterations of human C-reactive protein modified selectively in the absence of calcium. *Mol Immunol*. 1983; 20:1165–1175. [PubMed: 6656768]
19. Potempa LA, Siegel JN, Fiedel BA, et al. Expression, detection and assay of a neoantigen (Neo-CRP) associated with a free, human C-reactive protein subunit. *Mol Immunol*. 1987; 24:531–541. [PubMed: 2443837]

20. Kim IK, Ji F, Morrison MA, et al. Comprehensive analysis of CRP, CFH Y402H and environmental risk factors on risk of neovascular age-related macular degeneration. *Mol Vis*. 2008; 14:1487–1495. [PubMed: 18704199]
21. Seddon JM, Gensler G, Milton RC, et al. Association between C-reactive protein and age-related macular degeneration. *J Am Med Assoc*. 2004; 291:704–710.
22. Seddon JM, George S, Rosner B, et al. Progression of age-related macular degeneration: prospective assessment of C-reactive protein, interleukin 6, and other cardiovascular biomarkers. *Arch Ophthalmol*. 2005; 123:774–782. [PubMed: 15955978]
23. Robman L, Baird PN, Dimitrov PN, et al. C-reactive protein levels and complement factor H polymorphism interaction in age-related macular degeneration and its progression. *Ophthalmology*. 2010; 117:1982–1988. [PubMed: 20605213]
24. Seddon JM, Gensler G, Rosner B. C-reactive protein and CFH, ARMS2/HTRA1 gene variants are independently associated with risk of macular degeneration. *Ophthalmology*. 2010; 117:1560–1566. [PubMed: 20346514]
25. Mullins RF, Dewald AD, Streb LM, et al. Elevated membrane attack complex in human choroid with high risk complement factor H genotypes. *Exp Eye Res*. 2011; 93:565–567. [PubMed: 21729696]
26. Potempa LA, Yao Z-Y, Ji S-R, et al. Solubilization and purification of recombinant modified C-reactive protein from inclusion bodies using reversible anhydride modification. *Biophys Rep*. 2015; 1:18–33. [PubMed: 26942216]
27. Potempa, LA., Liao, HH., Crump, BL. Mutant protein and methods and materials for making and using it. US Patent. 5874238. 1999.
28. Chew EY, Clemons TE, Agrón E, et al. Ten-year follow-up of age-related macular degeneration in the age-related eye disease study: AREDS report no. 36. *JAMA Ophthalmol*. 2014; 132:272–277. [PubMed: 24385141]
29. Anderson DH, Mullins RF, Hageman GS, et al. A role for local inflammation in the formation of drusen in the aging eye. *Am J Ophthalmol*. 2002; 134:411–431. [PubMed: 12208254]
30. Bhutto IA, Baba T, Merges C, et al. C-reactive protein and complement factor H in aged human eyes and eyes with age-related macular degeneration. *Br J Ophthalmol*. 2011; 95:1323–1330. [PubMed: 21633121]
31. Wang H-W, Sui S-F. Dissociation and subunit rearrangement of membrane-bound human C-reactive proteins. *Biochem Biophys Res Commun*. 2001; 288:75–79. [PubMed: 11594754]
32. Khreiss T, József L, Potempa LA, et al. Conformational rearrangement in C-reactive protein is required for proinflammatory actions on human endothelial cells. *Circulation*. 2004; 109:2016–2022. [PubMed: 15051635]
33. Wu Y, Potempa LA, El Kebir D, et al. C-reactive protein and inflammation: conformational changes affect function. *Biol Chem*. 2015; 396:1181–1197. [PubMed: 26040008]
34. Nagy JA, Benjamin L, Zeng H, et al. Vascular permeability, vascular hyperpermeability and angiogenesis. *Angiogenesis*. 2008; 11:109–119. [PubMed: 18293091]
35. Johnson PT, Betts KE, Radeke MJ, et al. Individuals homozygous for the age-related macular degeneration risk-conferring variant of complement factor H have elevated levels of CRP in the choroid. *Proc Natl Acad Sci USA*. 2006; 103:17456–17461. [PubMed: 17079491]
36. Ji S-R, Le Ma, Bai C-J, et al. Monomeric C-reactive protein activates endothelial cells via interaction with lipid raft microdomains. *FASEB J*. 2009; 23:1806–1816. [PubMed: 19136614]
37. Wang M-Y, Ji S-R, Bai C-J, et al. A redox switch in C-reactive protein modulates activation of endothelial cells. *FASEB J*. 2011; 25:3186–3196. [PubMed: 21670067]
38. Bíró A, Rovó Z, Papp D, et al. Studies on the interactions between C-reactive protein and complement proteins. *Immunology*. 2007; 121:40–50. [PubMed: 17244159]
39. Chirco KR, Tucker BA, Stone EM, et al. Selective accumulation of the complement membrane attack complex in aging choriocapillaris. *Exp Eye Res*. 2015; 146:393–397. [PubMed: 26368849]
40. Mullins RF, Schoo DP, Sohn EH, et al. The membrane attack complex in aging human choriocapillaris: relationship to macular degeneration and choroidal thinning. *Am J Pathol*. 2014; 184:3142–3153. [PubMed: 25204844]

41. Vavvas DG, Daniels AB, Kapsala ZG, et al. Regression of some high-risk features of age-related macular degeneration (AMD) in patients receiving intensive statin treatment. *EBioMedicine*. 2016; 5:198–203. [PubMed: 27077128]
42. Karaca I, Ilkay E, Akbulut M, et al. Atorvastatin affects C-reactive protein levels in patients with coronary artery disease. *Curr Med Res Opin*. 2003; 19:187–191. [PubMed: 12803732]
43. Slevin M, Matou Nasri S, Turu M, et al. Modified C-reactive protein is expressed by stroke neovessels and is a potent activator of angiogenesis in vitro. *Brain Pathol*. 2010; 20:151–165. [PubMed: 19170684]
44. McLeod DS, Lefer DJ, Merges C, et al. Enhanced expression of intracellular adhesion molecule-1 and P-selectin in the diabetic human retina and choroid. *Am J Pathol*. 1995; 147:642–653. [PubMed: 7545873]
45. Mullins RF, Skeie JM, Malone EA, et al. Macular and peripheral distribution of ICAM-1 in the human choriocapillaris and retina. *Mol Vis*. 2006; 12:224–235. [PubMed: 16604055]
46. Skeie JM, Fingert JH, Russell SR, et al. Complement component C5a activates ICAM-1 expression on human choroidal endothelial cells. *Invest Ophthalmol Vis Sci*. 2010; 51:5336–5342. [PubMed: 20484595]
47. Stitt AW, O'Neill CL, O'Doherty MT, et al. Vascular stem cells and ischaemic retinopathies. *Prog Retin Eye Res*. 2011; 30:149–166. [PubMed: 21352947]
48. Ablonczy Z, Dahrouj M, Marneros AG. Progressive dysfunction of the retinal pigment epithelium and retina due to increased VEGF-A levels. *FASEB J*. 2014; 28:2369–2379. [PubMed: 24558195]
49. Liu G, Vogel SM, Gao X, et al. Src phosphorylation of endothelial cell surface intercellular adhesion molecule-1 mediates neutrophil adhesion and contributes to the mechanism of lung inflammation. *Arterioscler Thromb Vasc Biol*. 2011; 31:1342–1350. [PubMed: 21474822]
50. Waheed A, Sly WS. Membrane associated carbonic anhydrase IV (CA IV): a personal and historical perspective. *Subcell Biochem*. 2014; 75:157–179. [PubMed: 24146379]
51. Songstad AE, Wiley LA, Duong K, et al. Generating iPSC-derived choroidal endothelial cells to study age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2015; 56:8258–8267. [PubMed: 26720480]
52. Yuan X, Gu X, Crabb JS, et al. Quantitative proteomics: comparison of the macular Bruch membrane/choroid complex from age-related macular degeneration and normal eyes. *Mol Cell Proteomics*. 2010; 9:1031–1046. [PubMed: 20177130]
53. Crabb JW, Miyagi M, Gu X, et al. Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. *Proc Natl Acad Sci USA*. 2002; 99:14682–14687. [PubMed: 12391305]
54. Mullins RF, Johnson MN, Faidley EA, et al. Choriocapillaris vascular dropout related to density of drusen in human eyes with early age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2011; 52:1606–1612. [PubMed: 21398287]
55. Whitmore SS, Braun TA, Skeie JM, et al. Altered gene expression in dry age-related macular degeneration suggests early loss of choroidal endothelial cells. *Mol Vis*. 2013; 19:2274–2297. [PubMed: 24265543]
56. Nozaki M, Raisler BJ, Sakurai E, et al. Drusen complement components C3a and C5a promote choroidal neovascularization. *Proc Natl Acad Sci USA*. 2006; 103:2328–2333. [PubMed: 16452172]
57. Grossniklaus HE, Ling JX, Wallace TM, et al. Macrophage and retinal pigment epithelium expression of angiogenic cytokines in choroidal neovascularization. *Mol Vis*. 2002; 8:119–126. [PubMed: 11979237]
58. Montalvo V, Chan C-C, Gery I, et al. Complement deposits on ocular tissues adjacent to sites of inflammation. *Curr Eye Res*. 2007; 32:917–922. [PubMed: 18027167]
59. Whitcup SM, Sodhi A, Atkinson JP, et al. The role of the immune response in age-related macular degeneration. *Int J Inflam*. 2013; 2013:348092. [PubMed: 23762772]
60. Chen M, Xu H. Parainflammation, chronic inflammation, and age-related macular degeneration. *J Leukoc Biol*. 2015; 98:713–725. [PubMed: 26292978]
61. Gao J, Liu RT, Cao S, et al. NLRP3 inflammasome: activation and regulation in age-related macular degeneration. *Mediators Inflamm*. 2015; 2015:690243. [PubMed: 25698849]

62. Li H, Qiang Y, Wang L, et al. Effect of lipopolysaccharide on the characteristics of endothelial progenitor cells from bone marrow in mice. *Mol Med Rep.* 2014; 9:427–434. [PubMed: 24297111]
63. Martini JF, Piot C, Humeau LM, et al. The antiangiogenic factor 16K PRL induces programmed cell death in endothelial cells by caspase activation. *Mol Endocrinol.* 2000; 14:1536–1549. [PubMed: 11043570]
64. Schroeder A, Mueller O, Stocker S, et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol Biol.* 2006; 7:3. [PubMed: 16448564]
65. Kim D, Pertea G, Trapnell C, et al. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.* 2013; 14:R36. [PubMed: 23618408]
66. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics.* 2014; 30:923–930. [PubMed: 24227677]
67. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 2014; 15:550. [PubMed: 25516281]
68. Trapnell C, Roberts A, Goff L, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature Protoc.* 2012; 7:562–578. [PubMed: 22383036]

Figure 1.

Choroids from CFH high-risk donors have increased mCRP immunoreactivity. Donors were homozygous at the CFH allele for either the high-risk (A–C) or the low-risk (D–F) CFH genotype. Labelling with anti-mCRP antibody (B, E; purple, arrowheads) and anti-pCRP antibody (A, D; purple) was compared with labelling with secondary antibody only (C, F). A total of 25 eyes were evaluated and scored for each genotype (G). Scores were given based on the intensity of mCRP immunolabelling (H). RPE = retinal pigment epithelium; CC = choriocapillaris. Scale bar = 100 μ m.

Figure 2.

Choroids with the high-risk genotype have elevated mCRP. RPE/choroid tissue samples from heterozygous donors (HY) and those homozygous at the CFH allele for either the low-risk (YY) or the high-risk (HH) CFH genotype were analysed. (A) Total CRP ELISA was performed to compare total CRP levels between genotypes. Bars 1–4 = YY (n = 4); bars 5–10 = HY (n = 5); bars 11–15 = HH (n = 5). (B) Semi-native western blotting was performed using antibodies against pCRP (green, upper band) and mCRP (green, lower band). Lanes 2–5 = YY (n = 4); lanes 6–10 = HY (n = 5); lanes 11–15 = HH (n = 5). Relative band densities from the semi-native western blot were quantified for both pCRP (C) and mCRP (D). * $p < 0.05$.

Figure 3. mCRP localizes to sub-RPE deposits. Labelling with anti-mCRP antibody (A, C, E; purple) from three different donors compared with secondary antibody control (B, D, F). The black arrowhead indicates small druse (A); the asterisk indicates large hyaline druse (C); and the white arrowhead indicates large amorphous druse (E). Scale bar = 50 μ m.

Figure 4.

mCRP accelerates scratch closure and decreases TER in cultured endothelial cells. (A) Percent scratch closure for media control (blue; n = 12), pCRP (20 µg/ml; green; n = 24), and mCRP (20 µg/ml; red; n = 24). (B) TER assay with media control (blue; n = 9), pCRP (20 µg/ml; green; n = 9), and mCRP (20 µg/ml; red; n = 9). *p < 0.05; **p < 0.01; ***p < 0.005.

Figure 5.

Volcano plot summarizing RNA-Seq data for mCRP versus control RPE/choroid organ culture treatment. RNA-Seq analysis identified a list of 43 genes as differentially expressed (red points). The log₂ fold change values and p-values were calculated using DESeq2. Red points indicate genes that were called as significant (adjusted p-value < 0.05; at least 2-fold difference between groups) by both DESeq2 and Cuffdiff (see supplementary material, Table S2).

Figure 6.

Exogenous mCRP increases ICAM-1 after 12 h, while it decreases CA4 protein levels after 24 h in human choroid organ cultures. Representative western blots of human donor eye tissue after 12-h (A, C) or 24-h (B, D) treatment with mCRP or medium alone using anti-ICAM-1 (A, B; green, ~80 kDa) and anti-CA4 (C, D; red, 35 kDa) antibodies. Lanes representing mCRP-treated tissue are indicated with a '+'. Quantification of relative band densities is shown for ICAM-1 at 12 h (A, n = 18), ICAM-1 at 24 h (B, n = 8), CA4 at 12 h (C, n = 6), and CA4 at 24 h (D, n = 8). *p < 0.05.

Table 1

The biological processes most significantly affected by mCRP treatment are highlighted in the table at the top. The genes involved in the inflammatory response are presented in the table at the bottom

Biological process	Genes	Adjusted <i>p</i> -value
Inflammatory response	12	7.67E-08
Positive regulation of leukocyte migration	7	1.51E-08
Regulation of interleukin-6 production	6	5.23E-07
Positive regulation of nitric oxide biosynthetic process	5	5.23E-07
Response to tumour necrosis factor	7	1.59E-07
Regulation of cytokine biosynthesis process	6	2.34E-06

Gene name	Gene symbol	Fold change	Adjusted <i>p</i> -value
Chemokine (C-C motif) ligand 3	<i>CCL3</i>	5.00	7.19E-25
Selectin E	<i>SELE</i>	4.32	3.53E-44
Chemokine (C-C motif) ligand 4	<i>CCL4</i>	4.20	2.63E-21
Interleukin-1, alpha	<i>IL1A</i>	3.81	1.30E-15
Interleukin-1, beta	<i>IL1B</i>	3.36	1.18E-12
Interleukin-8	<i>IL8</i>	3.34	4.92E-20
Oxidized low-density lipoprotein (lectin-like) receptor 1	<i>OLR1</i>	3.23	9.49E-17
Tumour necrosis factor, alpha-induced protein 6	<i>TNFAIP6</i>	3.10	6.36E-17
Toll-like receptor 2	<i>TLR2</i>	2.27	2.46E-16
Intercellular adhesion molecule 1	<i>ICAM1</i>	2.14	2.79E-13
Interleukin-6	<i>IL6</i>	2.14	4.94E-05
Tumour necrosis factor, alpha-induced protein 3	<i>TNFAIP3</i>	2.08	6.13E-06