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Complement Regulation at Necrotic Cell Lesions Is Impaired by the Age-Related Macular Degeneration-Associated Factor-H His402 Risk Variant

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Age-related macular degeneration (AMD) is the leading cause of blindness in elderly populations in Western societies. Approximately 20 million individuals in the United States and Europe suffer from this sight-threatening disease (1, 2). Late disease stages present in two forms that both result in central visual loss. Geographic atrophy is caused by atrophy of retinal pigment epithelial (RPE) cells and subsequently photoreceptors in the macular area. Choroidal neovascularization is an abnormal growth of capillaries from the choiociapillaris through Bruch’s membrane and into the sub-RPE or subretinal space and is accompanied by increased vascular permeability and fragility. It can lead to subretinal hemorrhage, fluid exudation, lipid deposition, detachment of the retinal pigment epithelium from the choroid, fibrotic scars, or a combination of these findings. A hallmark of AMD and usually one of the first clinical symptoms is the presence of ocular drusen (3). Proteomic and histochemical analyses show that these extracellular deposits contain complement components and inflammatory proteins (4–6).

During the past 5 years, mutations in several complement genes have been linked to AMD. Genetic polymorphisms in genes coding for Factor H (7–9), Factor B, C2 (10), C3 (11), as well as a 84-kbp chromosomal deletion that results in the absence of the complement regulator CFHR1 and also of CFHR3 significantly influence AMD pathogenesis (12–14). One prominent AMD-associated polymorphism within the Factor H gene is a T → C substitution at nucleotide 1277, which results in a tyrosine (Y) to histidine (H) exchange at amino acid position 402. The Factor H risk variant His402 increases the risk for AMD ~2- to 4-fold for heterozygote and ~5- to 7-fold for homozygote individuals (7–9).

The human Factor H gene encodes two proteins: Factor H itself and the Factor H-like protein (FHL1). Both plasma proteins are synthesized in the liver but are also expressed locally (e.g., in RPE cells) (15). Factor H is composed of 20 consecutive protein domains, termed short consensus repeats (SCRs). FHL1, which is derived from an alternatively spliced transcript, represents the seven N-terminal SCRs of Factor H and has a unique C-terminal extension of four amino acids (16, 17). Both Factor H and FHL1...
include the AMD relevant residue 402 in SCR 7 and are major regulators of the alternative complement pathway (18). The two proteins act as cofactors for Factor I-mediated C3b inactivation and accelerate the decay of a preformed C3bBb convertase (19). Both regulators possess multiple binding sites for C3b, heparin, C-reactive protein (CRP), and bind to cellular surfaces. In this context, SCR 7 binds to the ligands heparin and CRP and mediates cell surface binding (20, 21). The nonrisk-associated variant Tyr402 of Factor H and of FHL1 bind more strongly to CRP than the His402 risk variants, suggesting a role of this AMD-associated polymorphism in ocular inflammation (15, 22–24).

CRP is an acute-phase protein, and the 125-kDa pentameric form (pentameric C-reactive protein; pCRP) is composed of five identical subunits, which are stabilized by calcium ions (25). pCRP is modified upon inflammation, surface attachment, oxidative stress, low pH, proteolytic cleavage, calcium depletion, and in vitro by heat or by urea treatment (26–28). Ultimately, pCRP dissociates into units of 23 kDa, termed monomeric C-reactive protein (mCRP). We and others have recently shown that Factor H and FHL1 bind to mCRP but not to pCRP and that mCRP recruits Factor H to the surface of apoptotic cells and apoptotic particles (29–31). Factor H attached to apoptotic surfaces controls complement progression, inhibits amplification of the complement cascade, enhances phagocytosis, and has anti-inflammatory activity (e.g., by reducing release of the proinflammatory cytokine TNF-α and the chemokine IL-8) (29).

The presence of CRP in drusen and ocular tissues is indicative for chronic local inflammation within retinal layers (32). Until now, the two CRP isoforms had not been discriminated in ocular tissues. However, individuals who are homozygous for the Factor H His402 risk variant show 2.5-fold higher CRP levels in the RPE-choroid layer compared with those of individuals homozygous for the nonrisk-associated Tyr402 variant (33). Persistent local inflammation near ocular drusen damages RPE cells and causes cell swelling, depigmentation, and changes in organelle distribution (34, 35). Ultrastructural and histochemical analyses indicate that during AMD, RPE cells overlying drusen degenerate because of necrosis (6, 36). Rupture of the plasma membrane is a characteristic feature for necrotic cell death and is followed by release of cytoplasmic contents in the form of DNA, histones, and other proteins, which initiate and further amplify local inflammation within the retina and attract blood-derived macrophages (37, 38).

In this study, we localize pCRP and mCRP within retinal tissues and show that mCRP is generated from pCRP at distinct patches on necrotic cells and colocalizes with the cell damage marker annexin V. The nonrisk-associated Tyr402 variant of Factor H forms strong complexes with mCRP and is recruited by mCRP to these necrotic lesions. Recruited Factor H maintains complement regulatory activity, efficiently inactivates complement, and blocks release of proinflammatory cytokine TNF-α. Reduced mCRP binding of the Factor H His402 risk variant results in complement activation, generation of inflammatory mediators, inflammation, and finally in pathology. In conclusion, we define a physiological, anti-inflammatory function of mCRP in retinal cells and demonstrate that the reduced interaction of mCRP with the risk variant of Factor H (and also FHL1) has functional effects that can explain certain aspects of AMD pathology.

Materials and Methods

Proteins and Abs

Serum-derived and recombinant human pCRP (Merck Biosciences, Schwabach, Germany) was used to generate mCRP by urea treatment (29). Goat and rabbit CRP antisera and the monoclonal mCRP Ab (clone CRP-8) were purchased from Sigma-Aldrich (Taufkirchen, Germany) and the monoclonal pCRP Ab (clone Mrrz-12) from Abds-online Gmbh (Aachen, Germany). Purified human Factor H, Factor I, C3b, and human Factor H antisera were obtained from Comptech (Tyler, TX). Alexa Fluor 555-, Alexa Fluor 488-, or Alexa Fluor 647-conjugated anti-goat, anti-rabbit, and anti-mouse IgGs were purchased from Invitrogen (Karloserue, Germany) and the HRP-conjugated anti-goat IgG, anti-rabbit IgG, and anti-mouse IgG from Dako (Hamburg, Germany). All functional assays were performed in Tris–cucumal buffer (140 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM Tris, pH 7.5) supplemented with 1% BSA. Complement Factor H derived from genotyped AMD patients was purified from plasma, and recombinant FHL1 was expressed in insect cells as described recently (15).

Patients

Patients were of Caucasian origin and were recruited at the Department of Ophthalmology of the University of Bonn and the University of Wuerzburg. This study was approved by the Institutional Review Board of the Friedrich Schiller University Jena, the University of Bonn, and the University of Wuerzburg. Genotyping of patients was performed as described by using genomic DNA isolated from blood leukocytes (15).

Cultivation of human RPE cell lines

The immortalized human RPE cell line ARPE-19 (ATCC no. CRL-2302) was cultivated as monolayers in a 1:1 mixture of DMEM and Ham's F12 medium (ATCC) supplemented with 10% FCS. Primary human RPE cells isolated by ScienCell Research Laboratories from human retina were purchased from Innoprot (reference no. P10873; Derio, Spain). Primary retinal cells were cultivated on poly-L-lysine–coated flasks in complete epithelial cell medium according to the manufacturer’s instructions (Innoprot). For detachment from their substratum, both the immortalized and the primary cells were incubated with Accutase (PAA, Pasching, Austria) for 5 min at 37°C. Necrosis of both cells was induced by incubating detached cells at 65°C for 1 h.

Flow cytometry

Necrotic ARPE-19 as well as necrotic primary RPE cells were incubated in 10 μg/ml pCRP, mCRP, Factor H, or FHL1, and binding of the different CRP isoforms was verified using a rabbit CRP antisera followed by the appropriate Alexa 488-labeled secondary antisera. Binding of the Factor H or FHL1 variants in the presence or absence of either mCRP or pCRP was detected with Factor H antisera and an Alexa 488-labeled goat antisera. In all experiments, 10,000 cells were routinely counted in a BD LSR II flow cytometer and analyzed with the FACSDiva (BD Biosciences, Heidelberg, Germany) and FlowJo software (Tree Star, Ashland, OR). Living intact cells were identified as propidium iodide-negative cells, and necrotic cells were identified as propidium iodide-positive cells.

Confocal microscopy

Surface binding of 10 μg/ml pCRP, mCRP, Factor H, or FHL1 to necrotic ARPE-19 as well as necrotic primary RPE cells was also visualized by confocal microscopy. Factor H and FHL1 were detected with polyclonal Factor H antisera followed by an Alexa 488-labeled goat antisera. The CRP isoforms were localized with mAbs to mCRP (CRP-8) or pCRP (Mrrz-12), respectively, followed by Alexa 488-coupled mouse antisera. Colocalization of mCRP and Factor H was assayed by incubating the cells first with mCRP followed by Factor H. After washing, monoclonal mCRP or polyclonal Factor H antisera was added, followed by the corresponding Alexa 488- or Alexa 555-labeled Abs. Cells were further stained with DAPI (Sigma-Aldrich) or annexin V labeled with allophycocyanin (BD Biosciences) and examined with a laser scanning microscope (LSM 510 META; Zeiss, Jena, Germany).

mCRP generation on the necrotic ARPE-19 surface

Conversion of 15 μg/ml pCRP attached to the surface of necrotic ARPE-19 cells was followed over 18 h. At the indicated time points (0.5, 2, 3, 4, and 18 h), cells were washed and stained with the specific mAb CRP-8. Addition of the Alexa 488-conjugated mouse antisera identified generated mCRP by flow cytometry and by confocal microscopy.
mCRP was bound with different amounts (0.5–1.5 μg/ml) to necrotic RPE cells, then either nonrisk or risk Factor H or FHL1 variants were added, followed by a 15-min incubation of C3b and Factor I. The deposition of C3b on the necrotic cell surface was assayed by flow cytometry using a monoclonal C3b Ab (Quidel, San Diego, CA) and an Alexa 488-labeled secondary mouse antiserum.

Immunohistochemistry

Immunohistochemistry was performed on ocular tissues derived from two normal human donor eyes (79 and 85 y, 2 female) and two eyes with a history of clinically documented early AMD (81 and 87 y, 2 female). The donor eyes were obtained at autopsy and were processed less than 15 h after death. Posterior eye poles were embedded in optimal cutting temperature compound and frozen in liquid nitrogen-cooled isopentane. Cryostat-cut sections (6 μm) were fixed in cold acetone, blocked, and incubated with mAbs to mCRP (CRP-8) or pCRP (Mrz-12), respectively, followed by Alexa 488-conjugated secondary antiserum (Molecular Probes, Eugene, OR). Nuclear counterstaining was performed with propidium iodide or DAPI (Sigma-Aldrich). Immunolocalization of mCRP and pCRP to Bruch’s membrane was confirmed by labeling of consecutive sections with an mAb against elastin (clone 10B8; Millipore, Schwalbach, Germany). In negative control samples, the primary Ab was replaced by PBS or equimolar concentrations of nonimmune mouse IgG.

Cytokine release

THP-1 monocytes were stimulated for 24 h with PMA (Sigma-Aldrich) to induce a macrophage phenotype (29). Necrotic ARPE-19 were cells incubated with the Factor H variants in the presence or absence of 1.5 μg/ml mCRP. After coincubation for 20 h, the culture supernatant was collected, and TNF-α content was determined using high-sensitivity human ELISA Set (Immuno Tools, Friesoythe, Germany).

Peptide spot analysis

Peptides that represent the SCR 7 region of complement Factor H/FHL1 were synthesized with a length of 12 and an overlap of 10 aa and coupled to a cellulose membrane (MultiPep; Intavis, Köln, Germany). After blocking, the membrane was incubated with 1 μg/ml mCRP for 2 h at room temperature. Bound mCRP was detected with monoclonal CRP-8 Ab followed by an HRP-labeled mouse antiserum. A second membrane treated in the same manner except that mCRP was absent was used as a negative control.

Structural modeling of SCR 7

The published structure of SCR 7 (Protein Data Bank, www.rcsb.org; code 2jgx) was used to locate the mCRP binding amino acids. The molecular graphic was created using the University of California San Francisco Chimera software (http://www.cgl.ucsf.edu/chimera).

Statistical analysis

PRISM GraphPad software was used for statistical analysis. Significant differences between two groups were analyzed by the unpaired Student t test. The results were considered statistically significant at *p ≤ 0.05, **p ≤ 0.01, or ***p ≤ 0.001.

Results

mCRP binding of Factor H is affected by the Tyr402 His variation

To assay the effect of the Factor H Tyr402 His variation on mCRP interaction, binding of pCRP or mCRP to the two immobilized Factor H variants was assayed by ELISA. Factor H, purified from plasma of genotyped AMD patients, is free of contaminants as demonstrated by SDSPAGE and silver staining (Supplemental Fig. 1A). The quality of mCRP generated from pCRP was characterized by ELISA based on the reactivity with mAbs specific for either pCRP or for mCRP (Supplemental Fig. 1B). mCRP, but not pCRP, bound dose-dependently to Factor H (Fig. 1A). mCRP binding to the nonrisk-associated variant was ~35–45% stronger compared with that of the risk variant. This effect is statistically significant for mCRP concentrations of 5 and 10 μg/ml (p < 0.01). Factor H–mCRP binding is specific as mCRP did not bind to immobilized BSA.

Cofactor assay

mCRP, but not pCRP, binds to Factor H by interaction with two linear mCRP binding motives within SCR 7. A, Factor H variants purified from genotyped AMD patients were immobilized on a microtiter plate, and pCRP and mCRP were applied in fluid phase and detected with a polyclonal CRP antiserum. mCRP, but not pCRP, bound both Factor H variants in a dose-dependent manner. mCRP bound up to 35–45% stronger than pCRP (Fig. 1B). The AMD-associated tyrosine at position 402 is colored green. Overlapping residues that are present in each peptide are shown in blue. The figure shows mean values of a triplicate assay of one representative experiment. The experiment was repeated three times with similar results. **p < 0.01. ∆ denotes the percent difference between the corresponding OD values for mCRP binding the presence of either Factor H Tyr402 or Factor H His402.

B, Amino acids within SCR 7 that mediate linear binding motives for mCRP were identified by peptide spot analysis. The cellulose membrane was incubated with purified mCRP, and bound mCRP was detected with the monoclonal CRP-8 Ab. mCRP bound to two regions each represented by four consecutive spots and indicated by black rectangles. One representative membrane of two independent experiments each is shown. C, Each binding region represents a linear sequence of 18 residues that are highlighted by a gray background in the selected sequence of SCR 7. Overlapping residues that are present in each peptide are shown in blue. D, The identified core sequences of the two identified linear mCRP binding motives (blue) are surface exposed and close to the relevant 402 residue (green) as revealed by molecular modeling. A, absorbance.

SCR 7 has two linear binding regions for mCRP

To identify residues within SCR 7 that contact mCRP, linear peptides with a length of 12 and an overlap of 10 residues were synthesized and spotted onto a membrane. This membrane was then incubated with mCRP, and bound mCRP was detected (Fig. 1B). mCRP bound to several peptides, which form two motives: motive I is represented by 399-NQNYGR (full sequence 389-408), motive II by 415-DVACHP (full sequence 405-423). The Tyr402 His polymorphism affects complement regulation.
pCRP and mCRP are present in the human retina–choroid complex and located in Bruch’s membrane and drusen

To analyze whether pCRP or mCRP are present in vivo, immunohistochemistry was performed using retinal–choroidal tissues from healthy individuals and from AMD patients. In tissues derived from healthy individuals, pCRP and mCRP reactivity was observed in the retinal pigment epithelium and at low levels within Bruch’s membrane (arrowheads). In AMD patients, pCRP staining is more pronounced and appears in Bruch’s membrane (arrowheads) and within drusen (arrows). C and D, mCRP showed similar reactivity as pCRP: weak staining in Bruch’s membrane (arrowheads) in healthy individuals (C) and stronger reactivity in tissues derived from AMD patients (D) predominantly in Bruch’s membrane (arrowheads) and within drusen (arrows). Autofluorescence of lipofuscin containing RPE cells appears yellow. The images are representative of two independent experiments. Scale bars, 200 μm. DR, drusen.

FIGURE 2. pCRP and mCRP are present in retinal–choroidal tissues of healthy individuals and AMD patients. Tissues derived from two human donor eyes with no or with clinically documented AMD were fixed in cold acetone, blocked, and incubated in a monoclonal mouse Ab to pCRP (MRz-12) or mCRP (CRP-8), respectively, overnight at 4°C. Bound Abs were visualized using an Alexa 488-conjugated secondary antiserum (green). Nuclear counterstaining was performed with propidium iodide (red). A, In the tissue derived from a healthy individual, pCRP is detected at low levels in Bruch’s membrane (arrowheads). B, In AMD patients, pCRP staining is more pronounced and appears in Bruch’s membrane (arrowheads) and within drusen (arrows). C and D, mCRP showed similar reactivity as pCRP: weak staining in Bruch’s membrane (arrowheads) in healthy individuals (C) and stronger reactivity in tissues derived from AMD patients (D) predominantly in Bruch’s membrane (arrowheads) and within drusen (arrows). Autofluorescence of lipofuscin containing RPE cells appears yellow. The images are representative of two independent experiments. Scale bars, 200 μm. DR, drusen.

FIGURE 3. Immunohistological localization of pCRP and mCRP in retinal–choroidal tissue of AMD patients. Tissues derived from two patients with clinical AMD were fixed in cold acetone, blocked, and incubated with a monoclonal mouse Ab specific for pCRP (MRz-12), mCRP (CRP-8), and a monoclonal mouse anti-elastin Ab (clone 10B8), respectively, overnight at 4°C. Bound Abs were visualized using an Alexa 488-conjugated antiserum (green) secondary Ab. Nuclear counterstaining was performed with DAPI (blue). A, Differentiation between specific pCRP fluorescence (green, I) and autofluorescence (red, II) of RPE cells in the same sections revealed specific pCRP immunoreactivity within drusen and Bruch’s membrane (green, III, arrows). B, Differentiation between specific mCRP fluorescence (green, I) and autofluorescence of RPE (red, II) revealed specific staining for mCRP within Bruch’s membrane and drusen (green, III, arrows). C, mCRP and elastin staining were performed in consecutive sections of ocular tissue derived from an AMD patient. Immunolocalization of mCRP to Bruch’s membrane (arrowheads) and drusen (arrow) (I). Immunolocalization of elastin to Bruch’s membrane (arrowheads) and to walls of choroidal blood vessels (double arrow) in a consecutive tissue section (II). Negative control in the absence of the primary elastin Ab reveals RPE autofluorescence but no reactivity of Bruch’s membrane (III). Corresponding brightfield images are shown in panels IV. BV, blood vessels; DR, drusen; RPE, retinal pigment epithelium; SC, sclera.
elastin, which represents a major structural component of Bruch’s membrane (Fig. 3C). By sequential application of different fluorescence emission filters on the same sections, autofluorescence in RPE cells (Fig. 3A, 3B, images II) could be differentiated from specific staining for both pCRP and mCRP (Fig. 3A, 3B, images I) within Bruch’s membrane and drusen (Fig. 3A, 3B, images III, arrows). Immunolocalization of pCRP and mCRP to Bruch’s membrane was further confirmed by costaining of consecutive tissue sections from AMD patients with an mAb to elastin. Both pCRP (data not shown) and mCRP reactivity was confirmed in drusen and Bruch’s membrane (Fig. 3C, image I) and colocalized with elastin in Bruch’s membrane (Fig. 3C, image II). Immunoreactivity for elastin was also observed in walls of choroidal blood vessels (Fig. 3C, image II, double arrow). Specific Ab binding was abolished when PBS or nonimmune serum was used instead of the primary CRP or elastin Abs revealing RPE autofluorescence only (Fig. 3C, image III).

**Binding of Factor H, pCRP, and mCRP to the surface of necrotic pigment epithelial cells**

To address whether the 402 sequence variation affects attachment of single proteins to damaged cellular surfaces, binding of both Factor H variants and also of the two CRP isoforms to necrotic ARPE-19 as well as primary RPE cells was assayed by flow cytometry. As single molecules, each Factor H variant and also the two CRP isoforms bound to the surface of necrotic ARPE-19 (Fig. 4A, 4B) and necrotic primary RPE cells with similar intensities (Fig. 4F, 4G).

Confocal microscopy was used to localize bound proteins at the surface of necrotic cells (Fig. 4C–E, 4H–J). Factor H and pCRP were equally distributed over the surface of necrotic ARPE-19 (Fig. 4C, 4D) and primary RPE cells (Fig. 4H, 4J) and the risk and nonrisk Factor H variants bound with equal intensities (data not shown). However, mCRP bound to specific sites of the necrotic retinal cells (Fig. 4E, 4J). The distribution of pCRP over the entire cell surface and the specific surface binding of mCRP was confirmed by three-dimensional imaging (Supplemental Fig. 2).

**Dissociation of pCRP into mCRP on the surface of necrotic ARPE-19 cells**

Prominent binding of mCRP to necrotic lesions suggested that mCRP is generated at sites of severe membrane damage. Therefore, pCRP was bound to the surface of necrotic ARPE-19 cells, and mCRP generation was followed over time by flow cytometry using the mCRP-specific mAb CRP-8. After binding pCRP to the surface of necrotic ARPE-19 cells, mCRP was already detectable after 0.5 h (Fig. 5A), mCRP reactivity increased with time, and after 18 h mCRP levels were significantly increased from background (p ≤ 0.007). On the surface of necrotic cells, gradual increase of mCRP reactivity over time was also confirmed by confocal microscopy (Fig. 5B–G). With this method, mCRP was detected after 2 h (Fig. 5D), and again staining increased with time (Fig. 5E–G).

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Factor H, pCRP, and mCRP bind to necrotic RPE cells. Binding of the nonrisk and risk variant of Factor H as well as of the two CRP isoforms to necrotic ARPE-19 as well as primary RPE cells was analyzed by flow cytometry. Bound Factor H was detected with polyclonal Factor H antiserum, pCRP and mCRP binding was analyzed with a polyclonal CRP antiserum that detects both forms. Unspecific Ab binding in the absence of proteins is shown by the dotted line. A and F, Factor H Tyr402 (black line) and Factor H His402 (gray line) bind to the surface of necrotic ARPE-19 (A) as well as primary RPE cells (F) with similar intensity. B and G, Also the two CRP isoforms, pCRP (black dashed line) and mCRP (gray dashed line), bound with similar intensity to necrotic ARPE-19 (B) and primary cells (G). Representative histogram profiles from five independent experiments are shown. C–E and H–J, Binding of Factor H, pCRP, or mCRP was assayed by confocal microscopy. The protective variant of Factor H was detected with a polyclonal Factor H antiserum together with an Alexa 647-conjugated secondary antiserum (red). Bound CRP forms were identified with mAbs (Mrz-12 for pCRP and CRP-8 for mCRP) together with an Alexa 488-labeled secondary antimouse serum (green). Cell nuclei were stained with DAPI (blue). Factor H (C, H) and pCRP (D, I) are evenly distributed over the surface of necrotic RPE cells. In contrast, mCRP binding (E, J) is restricted to a specific site. C–E and H–J are representative of five independent experiments. Scale bars, 20 μm.
mCRP recruits Factor H to sites of severe membrane damage

The binding of Factor H–mCRP complexes to specific surface spots suggested that these patches represent sites of severe membrane damage. Therefore, colocalization with annexin V, a marker for membrane damage, was assayed. Annexin V was distributed over the surface of necrotic ARPE-19 cells and showed a more intense staining at specific patches (Fig. 6E, red). Factor H–mCRP complexes bound preferentially to these specific patches (Fig. 6F, mCRP, green; and Fig. 6G, Factor H, turquoise) and colocalized with annexin V (Fig. 6H). Colocalization of mCRP and Factor H with annexin V and a similar surface distribution was also observed for necrotic primary RPE cells (data not shown).

Formation of such prominent necrotic surface patches that bind annexin V and also Factor H–mCRP complexes is a new observation and has to our knowledge not been reported before. We hypothesize that these sites of severe membrane alterations represent damaged, possibly ruptured membranes that release cytoplasmic content. Based on the specific binding properties and the selective recruitment of markers for cell damage and of the Factor H–mCRP complexes, we propose to term these sites urgent regulation of damage (URD) sites.

mCRP-assisted complement regulatory effects are influenced by the Tyr

Necrotic ARPE-19 cells lack membrane-bound complement regulators (Supplemental Fig. 3). To allow efficient opsonization of the surface by iC3b, these altered self surfaces attach fluid-phase regulators such as Factor H. As mCRP enhances C3b cofactor activity of Factor H (29), we asked if local complement regulation on such necrotic cells is mediated by Factor H–mCRP complexes and if this activity is affected by the Tyr His polymorphism. mCRP enhanced Factor H-mediated iC3b deposition in a dose-dependent manner (Fig. 7A). This effect was stronger for the nonrisk variant. The functional difference of 35% (at 1.5 μg/ml mCRP) between the nonrisk and risk variants was statistically significant (p ≤ 0.026). The same difference in iC3b inactivation was observed for Factor H–mCRP complexes in fluid phase (Supplemental Fig. 4A, 4B). Similar results were observed in the presence of primary RPE cells. Again, mCRP enhanced Factor H cofactor activity, and the two Factor H variants showed a functional difference of 17% at the highest mCRP concentration. However, the effect was not as prominent as for the ARPE-19 cell line (data not shown).

mCRP-mediated reduction of TNF-α release is affected by the Tyr

Factor H displays anti-inflammatory activity on apoptotic particles. Consequently, we asked 1) if this effect also applies for necrotic retinal cells, 2) if mCRP enhances this anti-inflammatory effect, and 3) if the effect is influenced by the Tyr His variation. Therefore, necrotic ARPE-19 cells were coated with one of the two Factor H variants and mCRP. These coated ARPE-19 cells

mCRP-mediated surface recruitment of Factor H is affected by the Tyr His variation

As both Factor H variants bound equally to necrotic surfaces, but mCRP preferentially binds the nonrisk variant of Factor H, we asked if the Tyr His variation may affect mCRP-mediated surface recruitment of Factor H to retinal necrotic cells. mCRP enhanced surface recruitment of both Factor H variants (Fig. 6A, compare the last column of each triplet with the first column) to ARPE-19 cells. However, binding of the nonrisk-associated Factor H variant was enhanced by ∼115% (p ≤ 0.0009), and binding of the risk variant was increased by ∼50%. pCRP did not affect Factor H surface binding (Fig. 6A, compare the second with the first column of each group).

mCRP-assisted recruitment of Factor H to the necrotic surface was visualized by confocal microscopy. To this end, mCRP was bound to the surface of necrotic ARPE-19 cells, and binding of the nonrisk-associated variant was analyzed (Fig. 6B). mCRP recruited Factor H and formed a prominent patchy pattern at the surface of necrotic cells. This “spot-like” staining in the presence of mCRP is clearly distinct from the even surface distribution observed for Factor H in the absence of mCRP (compare Fig. 6B with Fig. 4C). The staining pattern in the presence of mCRP suggests that mCRP recruits Factor H to specific sites on a necrotic cell. A very similar but not as pronounced effect was observed for necrotic primary RPE cells (Fig. 6C, 6D). In this case, mCRP enhanced surface recruitment of the protective Factor H variant by ∼45% (p ≤ 0.0057) and the binding of the risk variant by 14%. Again, pCRP had no significant effect on Factor H surface recruitment (Fig. 6D). mCRP-mediated Factor H recruitment occurred also on the surface of necrotic primary cells at a distinct spot of the cell (Fig. 6D).
were added to THP-1 cells, which were differentiated to macrophages by PMA to allow phagocytosis. After 20-h incubation, release of the proinflammatory marker TNF-α was assayed in the supernatant. Necrotic ARPE-19 cells induced TNF-α release (Fig. 7B, column 4). Coating the cell surface with mCRP alone did not affect surface binding of the Factor H variants (compare the second column of each group with the first column). C. On the surface of primary RPE cells, enhanced mCRP surface recruitment of the nonrisk Factor H variant by 45% and of the risk variant by 14%. Again, pCRP did not affect surface binding of the Factor H variants. Binding of both Factor H variants without preincubation of mCRP or pCRP was set to 100%. The percent enhancement (Δ) in the presence of mCRP was calculated by dividing the value obtained in the presence of the corresponding Factor H–mCRP complexes by the value of the Factor H binding alone. The data represent the mean ± SD for three independent experiments. **p < 0.01, ***p < 0.001.

Discussion

A tyrosine to histidine exchange at residue 402 of the complement regulator Factor H and of FHL1 increases the risk for AMD (7–9). In this study, we show how this polymorphism affects local complement control as well as anti-inflammatory processes on necrotic retinal cells. Reduced binding and recruitment of the risk variants by mCRP to specific necrotic cell lesions leads to functional differences that can develop into AMD. Necrotic cells lack membrane-bound regulators and therefore require surface-attached soluble inhibitors to allow opsonization with iC3b, to inhibit progression of complement beyond the C3 convertase step, and to allow efficient and noninflammatory clearance. The consequences of the Tyr402His variation were proved here for the established immortalized cell line ARPE-19 as well as on the surface of primary human RPE cells. On primary cells, mCRP showed indeed the same characteristics as on ARPE-19 cells but the effects were not as pronounced. Most likely because of their origin, primary cells are more sensitive to the applied necrotic procedures.

The role of the Tyr402His variation was also confirmed for FHL1, the second product of the Factor H gene. Similar to the Factor H variants, the nonrisk- and risk-associated variants of FHL1 showed differences in mCRP-directed effects (Supplemental Fig. 5A), mCRP-assisted recruitment to the surface of necrotic cells (Supplemental Fig. 5B–D), mCRP-assisted binding...
to URD sites (Supplemental Fig. 5E–I), and modified mCRP-mediated complement regulatory activities (Supplemental Fig. 5J, 5K). Thus, a second regulatory protein derived from the Factor H gene exists, whose function is impaired because of the Tyr402His variation. Factor H and FHL1 may act individually or in concert. However, the 402 sequence variation influences mCRP binding, resulting in inappropriate complement control on the necrotic surface, and mediates local inflammatory processes.

Currently, a specific physiological role for mCRP is emerging (29, 39). In this study, we identify both CRP isoforms in ocular tissues of AMD patients or healthy individuals (Fig. 2, Fig. 3). Enhanced mCRP staining in AMD tissues along Bruch’s membrane and also in drusen (Fig. 2D) supports the relevance of mCRP in vivo and particularly in AMD pathogenesis. mCRP is generated from pCRP on the surface of necrotic ARPE-19 cells (Fig. 5). Similarly, mCRP generation on the surface of activated platelets was recently reported (28).

A mCRP binding region in SCR 7 that contains relevant residue 402 is described for Factor H and also for FHL1 (29). Residue 402 is surface exposed and affects the intensity of mCRP interaction (Fig. 1) (15). In this study, we identify within SCR 7 two linear mCRP binding motives that are located either in direct (motive I) or in close vicinity (motive II) to residue 402 (Fig. 1D). When compared with the risk variants, the nonrisk-associated variants of Factor H and FHL1 bind mCRP with 40–50% higher intensity (Fig. 1A and Supplemental Fig. 5A). This difference influences mCRP-assisted surface recruitment to necrotic RPE cells (Fig. 6A, 6C, Supplemental Fig. 5D) and ultimately affects local complement regulation and release of the proinflammatory cytokine TNF-α (Fig. 7A, 7B, Supplemental Fig. 5J).

AMD is an inflammatory process (5) and is associated with necrosis of RPE cells that overlie drusen (6, 36). Progression of cellular necrosis results in morphological changes, loss of membrane integrity, membrane rupture, and release of cytoplasmic content (38). When not properly controlled and removed in a silent noninflammatory manner, necrotic material released from damaged cells activates complement and initiates local inflammation (34). It is of interest that histones that are released during necrosis were also identified in drusen (4). Necrotic ARPE-19 cells lack membrane-bound complement regulators, as demonstrated for CD46, CD55, and the terminal complement complex regulator CD59 (Supplemental Fig. 3). Consequently, a necrotic surface will activate complement. Complement activation to the level of the C3 convertase allows opsonization of the surface with C3b/C3bi. This enhances phagocytosis and efficient removal of cellular debris. However, progression of the cascade beyond the C3 convertase generates the potent inflammatory marker C5a, allows terminal complement complex formation, causes inflammation, and is therefore unfavorable. This scenario explains the need for surface-acquired regulators such as Factor H and FHL1 to block complement activation and to allow silent, noninflammatory removal of cellular debris (40, 41).

Factor H, FHL1, pCRP, and mCRP bind to the surface of necrotic RPE cells. Factor H (Fig. 4C, 4H), FHL1 (Supplemental Fig. 5C), and also pCRP (Fig. 4D, 4I) are evenly distributed over the surface of necrotic cells. However, mCRP locates to specific patches (Fig. 4E, 4J). Necrotic RPE cells expose altered self epitopes, such as phosphatidyl serine (42), that bind annexin V and provide specific binding sites for mCRP (Fig. 6E, Supplemental Fig. 5F). Based on the unique staining, requirement for complement control, and because of the unique binding features, we propose to term these patches URD sites. mCRP-assisted recruitment of Factor H and of FHL1 to URDs (Fig. 6A, 6C, Supplemental Fig. 5D) is affected by the tyrosine to histidine exchange at position 402. The reduced binding of the risk variant results in lower complement regulation and accumulation of debris. Thus, AMD patients with the risk allele at position 402 display a more “proinflammatory” intraocular environment than those who carry the nonrisk allele. Over time, apparently these differences extrapolate into higher levels of complement activation, inflammation, increased damage, and thus into pathology.
In summary, the Tyr402 His polymorphism of Factor H and FHL1 impair the mCRP-assisted complement control at necrotic surfaces (Supplemental Fig. 6). The crucial differences between the risk and nonrisk-associated variants affect 1) binding to differentiated human THP-1 macrophages mCRP, 2) mCRP-assisted recruitment to necrotic membrane lesions called URD sites, 3) local complement inhibition at the level of the C3 convertase, as well as 4) the release of the inflammatory cytokine TNF-α by human macrophages. Necrotic retinal epithelial cells bind the inflammatory marker pCRP and, at URD sites, the attached pCRP is dissociated into mCRP. Restriction of complement inactivation and anti-inflammatory processes cause accumulation of damaged material or debris and consequently translates into pathology of retinal tissue. Interfering and controlling these processes by complement-based anti-inflammatory intervention will ultimately provide a promising approach for AMD therapy.

Disclosures

The authors have no financial conflicts of interest.

References


