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John D. Lambris
Anthony P. Adamis
Editors

Inflammation and Retinal Disease: Complement Biology and Pathology

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Editors

Inflammation and Retinal Disease: Complement Biology and Pathology

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Preface

Numerous studies have pointed to the key role of complement in the pathogenesis of retinal disease, particularly age-related macular degeneration (AMD). Reports about new gene associations and links to other physiological pathways are emerging almost on a weekly base. Several promising clinical candidates covering a wide area of potential treatment applications are in the pipelines of both industrial and academic groups. This indicates an increasing interest in complement as a therapeutic target. In view of these exciting discoveries, scientists from around the world convened at the First Aegean Conferences Conference on Inflammation and Retinal Disease: Complement Biology and Pathology (June 10–17, 2007) in Crete, Greece, to discuss recent advances in this rapidly-evolving field. This volume represents a collection of topics on the functions of complement in eye diseases, pathophysiology, protein structures, and complement therapeutics discussed during the conference.

Our sincere thanks to the contributing authors for the time and effort they have devoted to writing what I consider exceptionally informative chapters in a book that will have a significant impact on the complement field. We would also like to express my thanks to Rodanthi Lambris for her assistance in collating the chapters and preparing the documents for publication and I gratefully acknowledge the generous help provided by Dimitrios Lambris in managing the organization of this meeting. Finally, I also thank Andrea Macaluso of Springer Publishers for her supervision in this book's production.

John D. Lambris
Anthony P. Adamis

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Chapter 1
The Case for Complement and Inflammation in AMD: Open Questions

Natalia Karagianni and Anthony P. Adamis

Abstract The complement cascade has been identified as a key factor in the pathogenesis of age-related macular degeneration (AMD). As a result, pharmacological modulation of the complement cascade is being investigated as a therapeutic strategy for AMD. The genetic data point to a triggering of the complement cascade, which subsequently cannot be damped down. Despite promising genetic, preclinical and immunolabeling data, important questions remain to be answered regarding the role of complement in the pathogenesis of AMD. The involvement of the complement cascade in the vision threatening stages of AMD, e.g. geographic atrophy and choroidal neovascularization, remain unknown. Additionally, the optimal component(s) of the complement cascade to be targeted for modulation still need to be identified. Answering these and other questions will provide investigators with a clear framework with which to evaluate progress in the field and help guide the development of future clinical therapeutics.

1 Introduction

Recent genetic data have identified an important role for the complement cascade in the pathogenesis of age-related macular degeneration (AMD). Drug candidates targeting the complement cascade have entered clinical development for the prevention and/or treatment of AMD. The scientific rationale for targeting the complement cascade will be reviewed, and gaps in our biological knowledge regarding complement and AMD will be identified.

Age-related macular degeneration (AMD) is the leading cause of blindness in the United States and Europe among the older individuals (Friedman et al. 2004). AMD is

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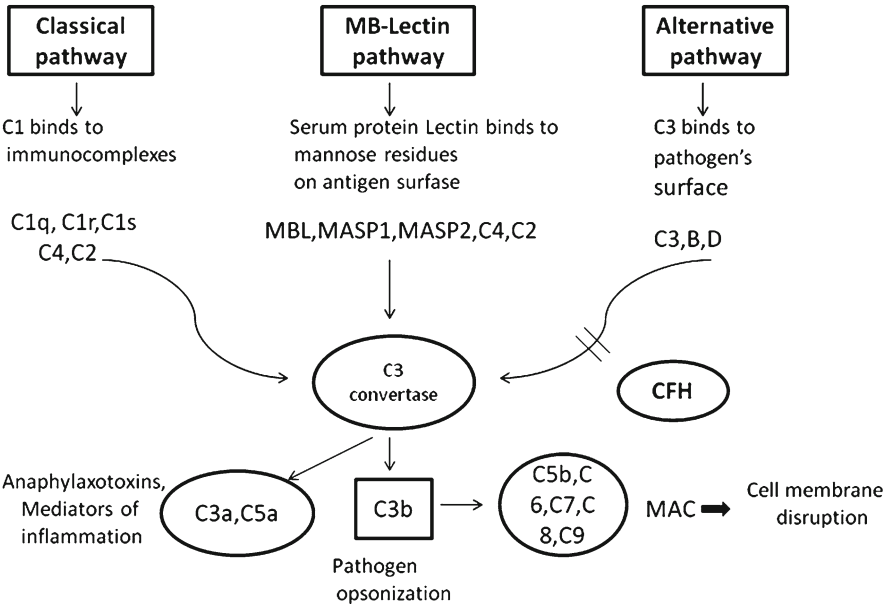

Fig. 1 Complement pathways. *CFH* complement factor H, *MAC* membrane attack complex, *MASP1–2* zymogen proteases, *MBL* mannose-binding lectin

associated with clinical depression in almost one-third of those who have the condition. Among individuals who have vision loss from AMD, 60% report significant declines in their ability to participate in everyday activities (Rovner and Casten 2002).

AMD is divided into dry and wet stages. Dry AMD refers to a heterogeneous graphic atrophy (GA), i.e. the focal loss of the outer neural retina. Wet AMD is characterized by choroidal neovascularization.

AMD begins with the presence of small drusen, which gradually become larger and finally coalesce to form confluent drusen, accompanied by retinal hyperpigmentation. At that crucial point the disease can progress to the stages associated with severe vision loss, choroidal neovascularization and/or geographic atrophy – stages termed "advanced AMD". Longitudinal studies recently demonstrated that confluent soft drusen are found in 100% of eyes at the site of later developing GA (Klein et al. 2008). The recently approved anti-VEGF drugs are very effective at treating choroidal neovascularization, but no pharmacological treatments are currently available to specifically treat geographic atrophy and the other pathologies of AMD (Fig. 1).

1.1 Epidemiology

Advanced age-related macular degeneration (geographic atrophy and/or choroidal neovascularization) affects more than 1.75 million individuals in the United States.

As the average lifespan of the US population increases, this number is projected to rise to almost three million by 2020 (Friedman et al. 2004).

It is estimated that 1–2% of the European and US populations, or approximately 1.6 million eyes, are affected with geographic atrophy and one million are affected with choroidal neovascularization. If one eye has advanced AMD (geographic atrophy and/or choroidal neovascularization), the second eye has a 43% chance of progression to advanced AMD within 5 years (Bressler et al. 2003).

A breakthrough came in 2005 when four high profile papers on the genetics of AMD independently confirmed a single nucleotide polymorphism in complement factor H (CFH) that was tightly linked to an increased risk of developing both early and late AMD (Haines et al. 2005; Klein et al. 2005; Edwards et al. 2005; Hageman et al. 2005). The Tyr402His substitution in CFH, the major negative regulator of the alternative complement pathway, was shown to be responsible for approximately 50% of AMD cases.

The mutually confirmatory nature of these results linked the pathogenesis of AMD to the complement cascade and its attendant inflammation. These results have stimulated the development of complement inhibitors for the treatment of AMD. However, as these compounds enter clinical development, a significant number of questions regarding the role complement in AMD remain unanswered.

Since the initial publication of the CFH genetic data, other factors in the complement pathway have been implicated. The genetic data all point to a triggering of the complement cascade, particularly the alternative pathway, which subsequently cannot be sufficiently damped down. How this phenomenon leads to AMD remains unknown.

A brief review of the data supporting the role of complement in AMD follows.

2 Drusen

Immunolabeling data have shown that almost all components of the complement cascade can be localized to Bruch's membrane and drusen in humans with AMD. Notably, the membrane attack complex (MAC; C5b-9) and the inflammatory chemotaxins, i.e. the anaphylaxotoxins C3a and C5a are present in early AMD (Anderson et al. 2002; Nozaki et al. 2006). Separate studies have shown occasional monocyte-like cells invading drusen, which may be causally related to the proximal complement deposition (Anderson et al. 2002; Hageman et al. 2001).

3 Geographic Atrophy

There is no direct evidence that implicates complement in the pathogenesis of geographic atrophy. Detailed transmission electron microscopy studies have demonstrated that multinucleated giant cells are in close association with the boundaries

of geographic atrophy (Penfold et al. 1986).However it is not known if these inflammatory cells are causal in the pathogenesis of geographic atrophy. More recently, Weber and colleagues compared the presence of the at risk CFH polymorphism with the growth rate of geographic atrophy and found no association with disease progression (Scholl et al. 2009). Because the CFH polymorphism is associated with drusen, they speculated that CFH (and complement in general), may play a role earlier in the disease, i.e. during the initiation and/or progression of drusen, but perhaps not directly in the onset and progression of geographic atrophy.

4 Choroidal Neovascularization

Both indirect and direct data implicate the complement cascade (and inflammation) in the pathogenesis of choroidal neovascularization. Hans Grossniklaus (2000) has shown that macrophages co-localize to areas of active choroidal neovascularization in human eyes with wet AMD. Direct data have been obtained in animal models. When CNV is induced with a laser, macrophages participate in the pathological neovascularization. When the inflammatory cells are selectively eliminated via CD18 or ICAM-1 deficiency, CNV is reduced by 50–70%, arguing that the inflammatory cells somehow enhance the CNV process in this model. Similarly, in rodent models of oxygen induced retinopathy and corneal neovascularization, the selective elimination of inflammatory cells potently suppressed the growth of new pathological vessels. Taken together, these animal data suggest that inflammation contributes the extent and severity of pathological neovascularization.

More recently, the direct role of complement-mediated inflammation in CNV was confirmed. In a study by Ambati and colleagues, C5aR and C3aR knock-out mice demonstrated a marked inhibition of laser-induced CNV (Nozaki et al. 2006). In separate experiments, intravitreal injections of small molecule C5aR inhibitor dose-dependently suppressed CNV in the laser-induced CNV model (Schnatbaum et al. 2006). The totality of the animal data suggest a direct role for complement in choroidal neovascularization, however the confounding variable of laser-induced injury tempers any firm conclusions.

In animal models of complement-mediated non-ocular diseases, e.g. neutropenia, immune complex peritonitis, renal fibrosis, myocardial infarction and kidney transplantation rejection, C5aR inhibition has shown a beneficial effect. These data suggest that the complement-mediated inflammation participates in the pathogenesis of the above-listed diseases. However, other complement-mediated diseases appear to be caused primarily by the membrane attack complex (MAC), one example being paroxysmal nocturnal hemoglobinuria (Parker 2008). Thus, it remains to be clarified which action(s) of the complement cascade are operative in the pathogenesis of AMD: (1) inflammation, (2) opsonization, and/or (3) MAC formation.

In spite our gaps in knowledge, the first clinical trials of complement inhibition in AMD have begun. Potentia Pharmaceutics C3 convertase inhibitor, compstatin a.k.a. POT-4, successfully completed a Phase I clinical trial in advanced wet AMD

patients in March 2009. The compound demonstrated some early signs of bioactivity and appeared to have an adequate safety profile. Ophtotech's ARC1905, a C5 blocking aptamer, also entered Phase 1. Both compounds inhibit complement-mediated inflammation and MAC formation.

More recently, new data have highlighted the incomplete nature of our knowledge regarding the role of complement in AMD. Coffey et al. (2007) showed that 2-year-old CFH deficient (cfh−/−) mice unexpectedly developed a decrease in electron-dense material and a thinning of Bruch's membrane. These findings are surprising as they are the opposite of what is seen in human AMD. As noted above, Scholl et al. (2009) studied patients with geographic atrophy and showed that those with the Tyr402His CFH polymorphism did not have an increased rate of GA progression. Their data suggest that the CFH "at risk" genotype contributes to the increased risk of late AMD largely or entirely through its impact on precursors, such as drusen and other RPE and Bruch's membrane changes. The authors propose that complement triggers drusen formation followed by separate disease process that leads to GA.

Since the primary source of CFH is the liver, Greg Hageman and colleagues are conducting a study in liver transplant recipients to assess AMD progression in patients possessing wild type CFH who receive a liver with the "at risk" CFH polymorphism, and vice versa. These data may yield important insights regarding the role of CFH in the various stages of AMD. The immunosuppression transplant patients receive during liver transplantation is a potentially confounding variable, however all groups of patients will presumably receive similar amounts of immunosuppressive drugs.

Eculizumab (Soliris, Alexion Pharmaceuticals) is the first complement inhibitor approved by the FDA. In its pivotal phase 3 trials, eculizumab proved effective in patients with paroxysmal nocturnal hemoglobinuria, a complement mediated disease. Eculizumab is a monoclonal antibody that binds to and blocks C5 bioactivity. At Bascom Palmer, Phil Rosenfeld is conducting a small prospective study quantifying drusen and GA progression in cohorts of patients receiving eculizumab or a placebo control. This timely study may provide important data about the direct causal role of the complement cascade in drusen formation/progression and geographic atrophy progression. However, it is not clear if this 1-year study in 40 patients has sufficient statistical power to detect a small treatment effect.

The aforementioned data highlight that with our current state of knowledge, the appropriate stage of AMD to target with complement cascade modulation remains unknown. Moreover, the component(s) of the complement cascade to be targeted for modulation still need to be identified, i.e. the respective roles of complement-mediated opsonization, inflammation and MAC formation in the pathogenesis of AMD remain unknown.

In evolutionary terms, complement cascade, especially the alternative pathway, is the most ancient part of our immune system. Complement related proteins are present in sponges and are highly conserved throughout evolution. Chronic blockade of this part of the immune system could in theory make patients more vulnerable to infections and other diseases. More specifically, the intravitreal injection of a complement-blocking drug could potentially predispose patients

to endophthalmitis – a serious sight-threatening bacterial infection of the eye. It is well known that even under sterile conditions, the inadvertent injection of bacteria occurs in a subset of patients. Experimental studies have shown that guinea pigs are prone to unchecked bacterial replication in the vitreous when the complement system is specifically inhibited (Giese et al. 1994). Endophthalmitis could therefore represent a ocular "stress-phenotype" that is more likely following the intravitreal injection of a complement inhibitor.

From a drug development perspective, it is also important to learn if the complement cascade is mechanistically linked to other known or suspected pathways in the pathogenesis of AMD, such as VEGF-driven choroidal neovascularization, lipofuscin deposition and/or Chlamydia infection (Nozaki et al. 2006; Zhou et al. 2006). A knowledge of disease pathway interactions will help discern if additive or synergistic responses are likely with combination therapy.

These and other questions should catalyze future studies on the role complement in AMD, as investigators work make the most of the important genetic clues that have been uncovered. As research continues to unravel the biology of AMD, complement inhibition may prove to be an important pharmacological approach in the treatment of this visually debilitating disease.

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Chapter 2
The Role of Complement in AMD

Peter F. Zipfel, Nadine Lauer, and Christine Skerka

Abstract Age related macular degeneration (AMD) is a common form of blindness in the western world and genetic variations of several complement genes, including the complement regulator Factor H, the central complement component C3, Factor B, C2, and also Factor I confer a risk for the disease. However deletion of a chromosomal segment in the Factor H gene cluster on human chromosome 1, which results in the deficiency of the terminal pathway regulator CFHR1, and of the putative complement regulator CFHR3 has a protective effect for development of AMD. The Factor H gene encodes two proteins Factor H and FHL1 which are derived from alternatively processed transcripts. In particular a sequence variation at position 402 of both Factor H and FHL1 is associated with a risk for AMD. A tyrosine residue at position 402 represents the protective and a histidine residue the risk variant. AMD is considered a chronic inflammatory disease, which can be caused by defective and inappropriate regulation of the continuously activated alternative complement pathway. This activation generates complement effector products and inflammatory mediators that stimulate further inflammatory reactions. Defective regulation can lead to formation of immune deposits, drusen and ultimately translate into damage of retinal pigment epithelial cells, rupture of the interface between these epithelial cells and the Bruch's membrane and vision loss. Here we describe the role of complement in the retina and summarize the current concept how defective or inappropriate local complement control contributes to inflammation and the pathophysiology of AMD.

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1 Age-Related Macular Degeneration

1.1 The Disease

Age related macular degeneration (AMD) is the leading cause of blindness in the elderly population, especially in the Western World. Today, more than 20 Mio individuals over 50 years of age show early signs of this sight-threatening disease (Congdon et al. 2004; Pascolini et al. 2004). The actual demographic development predicts an increase in the number of elderly people and thus a higher number of people at risk by about 50 % (Friedman et al. 2004).

In the early stage of the disease immune deposits, which are termed drusen, develop between the Bruch's membrane (BM) and adjacent retinal pigment epithelial cell (RPE) layer (Bird et al. 1995). Ongoing progression of the inflammatory reactions and the disease enhances size and the number of drusen and result in two severe forms: geographic atrophy (GA) leads to death of macula surrounding photoreceptors that overly degenerated RPE cells. Choroidal neovascularisation (CNV) is characterized by the growth of blood vessels into the retinal layer which leak fluid or bleed. Both forms result in a complete loss of central vision (de Jong 2006).

AMD is a multifactorial disease which is caused by several genetic factors, by environmental factors and disease susceptibility is also influenced by age and ethnic background (Vingerling et al. 1995; Klein et al. 2004).

1.2 AMD: A Chronic Inflammatory Disease

The pathogenesis of AMD is subject of intensive research and recent reports showed that early as well as advanced stages of AMD are caused by defective complement activation and local inflammation (Johnson et al. 2001). At present additional concepts for AMD pathophysiology are discussed, which however are not exclusive to the initial complement and inflammation hypothesis. These explanations are based on inefficient or defective transport/diffusion of nutrients from the choroids via the Bruch's membrane to the photoreceptors, or inappropriate reverse transport of waste products from photoreceptors to the choroids (Zarbin 1998). Consequently debris may accumulate along the interface of the RPE cells and the Bruch's membrane, providing an activator surface that allows complement activation. Also in this concept defective or inefficient complement regulation may cause further amplification of the complement cascade and inflammation. Both concepts may explain how inappropriate control and inhibition of spontaneous or triggered complement activation causes progression and amplification of the complement cascade and consequently the generation of inflammatory activation compounds in form of C3a and in particular of the potent inflammatory marker C5a (Scholl et al. 2008). If low level complement activation persists over weeks, months and even years, already a minor change in the composition of one regulatory component may

cause deregulation and damage. This continuous imbalance can then progress into pathophysiology, in manifestation of drusen and degeneration of the RPE cell layer and of the overlying photoreceptors.

AMD is associated with complement activation or deregulation of the spontaneously initiated alternative complement pathway leading to local release of inflammatory activation products and to local inflammation, which relate to the pathogenesis of the disease (Donoso et al. 2006). This concept is further confirmed by immunohistochemical analyses and proteome assays, which identified proteins and components associated with complement activation and inflammation in drusen. Multiple complement components, regulators, complement activation products and inflammatory proteins are identified in drusen, including C3, C3d, the terminal components C5, C6, C7, C8 and C9, terminal complement regulators vitronectin and clusterin, apolipoproteins apoA1, apoA4 and apoE as well as thrombospondin, serum amyloid A (SAP-A) and SAP-P (Anderson et al. 2002; Crabb et al. 2002; Li et al. 2006a). This composition demonstrates the association of complement and complement regulators in the process of drusen formation and likely also in the associated inflammatory reactions. The activated complement systems triggers further events and which ultimate cause formation of drusen, cell damage of the RPE cells and visual loss (Hageman et al. 2001).

Most or all of these proteins that are identified by immunohistochemistry and proteome analyses are expressed locally, e.g. by RPE cells and are present in the choroids. Thus explaining or confirming a local role of complement and inflammation in the pathogenesis of AMD. Immunohistochemical analyses also identified the inflammatory marker C-reactive protein (CRP) in drusen and in deposits formed along the Bruch's membrane (Anderson et al. 2002; Laine et al. 2007; Skerka et al. 2007). Based on immunoblot assays the local level of C-reactive protein (CRP) in the choroidal stroma was about 2.5-fold higher in individuals with the Factor H risk variant (Johnson et al. 2006). These increased CRP levels in the choroids of individuals at risk are indicative for a chronic inflammation. Upon inflammation, infection or tissue damage CRP plasma levels increase substantially, from minimal, undetectable levels to plasma concentrations of more than 500 mg/l (Pepys and Hirschfield 2003). CRP, is a member of the pentraxin protein family and this 125 kDa is composed of five identical subunits, which are stabilized by calcium ions (Volanakis 2001; Casas et al. 2008). In addition a monomeric 25 kDa form of CRP exists (Potempa et al. 1987; Ji et al. 2007; Mihlan et al. 2009). The exact biological functions of pCRP and mCRP are currently unclear.

2 Age-Related Macular Degeneration: A Genetic Disorder

Population based analyses, twin studies (Hammond et al. 2002; Seddon et al. 2005) and familial aggregation analyses (Seddon et al. 1997) suggested that AMD is a heritable disease and that the majority of late AMD cases has a specific genetic background. Over the last years evidence accumulated that AMD is caused by

genetic factors. Initiated by the Human Genome Project in 2005, four independent genome-wide linkage studies identified two major chromosomal loci that confer major risk for this retinal disease and that account for approximately 50% of cases (Edwards et al. 2005; Haines et al. 2005; Klein et al. 2005; Rivera et al. 2005). One chromosomal region is located in human chromosome 1q31, which includes the Factor H gene cluster, and the second region is located on human chromosome 10q26 which covers the two closely located genes ***ARMS2*** (**a**ge-**r**elated **m**aculopathy **s**usectibility **2**) and ***HTRA1*** (**h**igh-**t**emperature **r**equired factor **A1**) (Dewan et al. 2006; Yang et al. 2006).

The region on human chromosome 1q31 includes the gene representing the complement regulator **Factor H** and the five CFHRs (**C**omplement **Factor H R**elated genes) CFHR1 to CFHR5 (Rodriguez de Cordoba et al. 2004). Within the *Factor H gene* numerous relevant single nucleotide polymorphisms (SNPs) were identified by SNP genotyping, including regions within the promoter-, the coding- and also within the non coding intronic regions (Li et al. 2006b). The major risk variant SNP rs1061170 is located in exon 9, and translates on the protein level at position 402 of both Factor H and FHL1 in the exchange of the amino acid tyrosine (Y) to the risk variant histidine (H). The H402 variant increases the risk for AMD about twofold to fourfold for heterozygotes cases and about threefold to sevenfold for homozygote individuals. Subsequent genetic studies confirmed the high association of the Y402H polymorphism in population and different ethnic groups worldwide (Souied et al. 2005; Okamoto et al. 2006; Seitsonen et al. 2006; Simonelli et al. 2006), and revealed that the H402 risk variant confers a similar risk for development of drusen, and the two severe forms of GA and CNV (Magnusson et al. 2006). Additional SNPs associated with exudative age related macular degeneration were identified in the promoter region of Factor H (position -257; SNP rs3753394); in exon 2, resulting in the exchange at residue 62 of isoleucine (I) to valine (V) (I62V; SNP rs800292), in exon 7 changing the codon but maintaining the alanine (A) residue (A307A; SNP rs1061147) and two which are located within the intervening sequence between exon 15 and exon 16 (SNPs rs380390 and rs1329428) (Chen et al. 2006).

A Chromosomal deletion of a 84-kbp fragment directly downstream of the *Factor H gene*, that includes the genes coding for CFHR1 and CFHR3 has a protective effect in AMD (Hughes et al. 2006; Spencer et al. 2008a; Schmid-Kubista et al. 2009). Case control studies revealed the complete absence of both genes comprise 0.8–1.1% of cases and 2.6–5.7% of the age-matched controls (Hageman et al. 2006).

Additional complement genes associated and linked to AMD include the classical pathway component **C2,** as well as the alternative pathway protein **Factor B** which are both located in close arrangement on human chromosome 6p21 (Gold et al. 2006). The L9H and R32Q variant in Factor B are in nearly complete linkage disequilibrium with the E318D or rs547154 SNP9 in intron 10, respectively of C2. All four polymorphisms are highly protective for AMD. In addition a common polymorphism, i.e. rs2230199 in the ***C3*** *gene*, which is encoded on chromosome 19p13 is strongly associated with AMD (Yates et al. 2007; Spencer et al. 2008b). This variation of C3 that results on the protein level in an arginine (R) to glycine (G) exchange

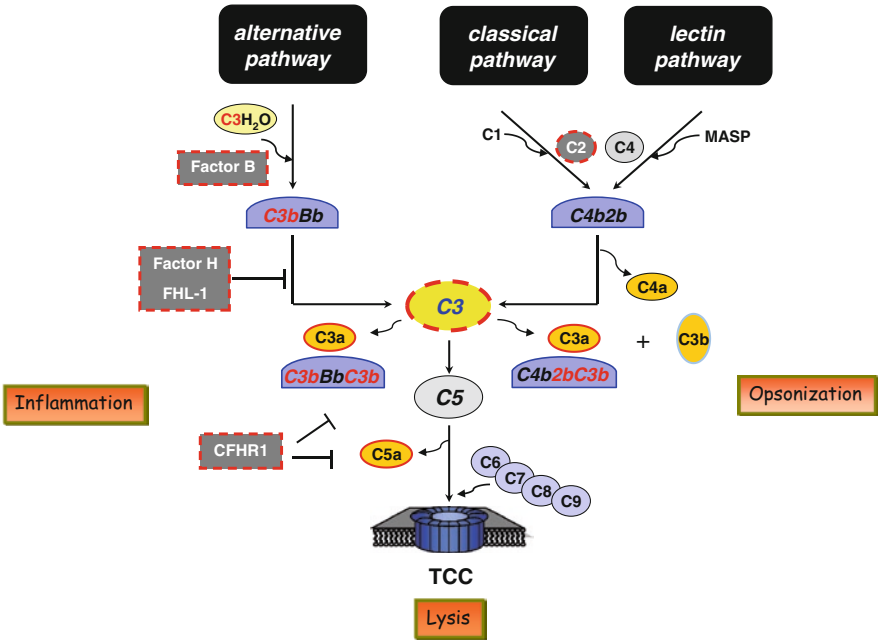

Fig. 1 Complement genes associated with AMD. The complement cascade is initiated by three separate pathways: the alternative, the lectin and the classical pathway. The three major effort functions of the activation completed cascade result in inflammation (effector compounds C3a and C5a), lysis (terminal complement complex, TCC) and opsonization (effector compound surface deposited C3b). Complement proteins that are associated with AMD are indicated by *red dashed lines* include the regulators of the alternative pathway convertase C3bBb: Factor H and FHL1, the C5 convertase and TCC inhibitor CFHR1, Factor B, C3 and C2. The enzymes that are generated and assembled upon complement cascade activation are shown with a blue background

at position 102 (R102G) increases the risk for AMD about twofold for heterozygote and about threefold for homozygote individuals. The association of complement genes in the pathogenesis of AMD further strengthens the concept that the human complement system is involved in the pathogenesis of AMD (Fig. 1).

The second disease associated locus on human chromosome 10q26 includes two separate genes. ***ARMS2*** (also termed LOC387715) which encodes a hypothetical protein. The AMD-associated SNP rs10490924 on exon 1 of the *ARMS2 gene* reveals an estimated risk of 2.6 for heterozygous und 7.0 for homozygous individuals (Rivera et al. 2005). The SNP results in a non-synonymous A69S alteration in the corresponding protein (Fritsche et al. 2008). Recent studies confirmed that this polymorphism in the ARMS2 gene is highly associated with AMD. The second high-susceptibility gene within the 10q26 region comprises the SNP rs11200638 within the ***HTRA1*** gene that is suggested to present a promoter variant of the heat shock serine protease (Dewan et al. 2006; Yang et al. 2006).

3 Effect of the Reported SNPs for Protein Function

3.1 Factor H and Other Complement Proteins

The genetic associations summarized above demonstrate that Factor H, FHL1, CFHR1, CFHR3, C2, Factor B and C3 are involved with AMD development and they show that complement plays a key role in the disease. Obviously sequence exchanges in complement genes and in the encoded complement proteins and regulators disturb the delicate balance of complement activation vs. inhibition and result in activation and inflammation (Zipfel and Skerka 2009).

3.1.1 Factor H and FHL1

The human *Factor H gene* encodes two proteins, **Factor H**, which is composed of 20 SCR domains, and the **Factor H-like protein** (FHL1) (Zipfel and Skerka 1999; Jozsi and Zipfel 2008). FHL1 is derived from an alternatively spliced transcript and the secreted protein is composed of the first seven N-terminal domains of Factor H including codon 402 in SCR 7 and exhibits a unique C-terminal extension of four amino acids (Misasi et al. 1989). Both Factor H and FHL1 are complement regulators that act on the level of C3 convertase and control formation and fate of C3 and C3 convertase C3bBb (Pangburn et al. 1977; Kuhn and Zipfel 1996). Both regulators have multiple binding sites for C3b, Heparin, C-reactive protein (CRP) and binding sites for cellular and biological surfaces. In this context SCR 7 mediates binding to heparin, cell surfaces and CRP (Jarva et al. 1999; Giannakis et al. 2003).

The AMD-associated tyrosine to histidine exchange at position 402 of both Factor H and FHL1 affect the binding intensity to heparin and CRP (Clark et al. 2006; Laine et al. 2007; Sjoberg et al. 2007; Skerka et al. 2007; Yu et al. 2007; Ormsby et al. 2008). The protective variants of both Factor H and FHL1, carrying Y402, bind stronger and the risk variants with H402 bind with lower affinity to their ligands. This differences in binding are explained by the alteration of the distribution of positively charged amino acids by the H402 which are essential for a proper heparin binding over SCR 7 (Prosser et al. 2007; Ormsby et al. 2008). However, differences in heparin affinity of the two allotypes were not reported consistently, what is explained to be glycosaminoglycan specific (Herbert et al. 2007).

The reduced heparin binding of the risk variant translates into lower binding to cellular surfaces, e.g. of RPE cells and results in an inefficient complement regulation at the cell surface (Skerka et al. 2007).

The exchange at amino acid position 402 also affects interaction with the inflammatory marker CRP. Using either intact Factor H, recombinant FHL1 or recombinant fragment SCRs 6–8 the protective variants always showed stronger binding, and the risk variants showed consistently reduced binding to CRP. Individuals who are homozygous for the risk form H402 show 2.5-fold elevated CRP immunoreactivity throughout the choroid and extracellular deposits along the Bruch's membrane as

demonstrated by immunohistochemistry (Johnson et al. 2006). Conversely, AMD patients that carry the protective Y402 phenotype show lower CRP immunoreactivity in the choroidal stroma, drusen as well as basal deposits. Deposition of CRP in drusen or subRPE deposits is a biomarker for chronic inflammation in RPE/chroid complex (Ross et al. 2007). This suggests that the Y402H polymorphism affects Factor H-mediated CRP function and plays a role in local ocular inflammation and cellular injury of RPE cells provoked by complement activation.

One major function of the activated complement system is to identify and tag modified self-cells such as apoptotic particles and necrotic cells, to allow non-inflammatory clearance (Zipfel and Skerka 2009). CRP marks damaged cells and tissues by binding to constituents such as DNA or phosphocholine that are exposed on injured cells. CRP binds the inhibitor Factor H (Ji et al. 2006a, b). Binding of this central soluble complement regulator is relevant on the surface of apoptotic and necrotic cells. CRP activates the classical complement pathway resulting in formation of the C3 convertase which generates C3b and causes C3b surface deposition and opsonization. However, by binding the inhibitor Factor H further progression of the cascade, amplification, C5 convertase formation and terminal pathway activation is inhibited (Gershov et al. 2000; Mihlan et al. 2009). Recently we showed that the monomeric form of CRP, mCRP binds Factor H and increases Factor H inhibitory action on the surface of apoptotic self cells (Mihlan et al. 2009). Factor H has three binding sites for mCRP and one binding site is located in the AMD-associated SCR 7 of both Factor H and FHL1 (Mihlan et al. 2009). Consequently uptake and removal of apoptotic particles was enhanced and bound Factor H showed a potent anti-inflammatory effect.

Recently also a functional difference was reported for the N-terminal located isoleucin (I) to valine (V) exchange at amino acid position 62 of Factor H and FHL1. The I62 variant is associated with the protective form for AMD and most likely has a higher thermal stability (Hocking et al. 2008). In addition Tortajada et al. showed that the protective I62 variant exhibits an increased affinity for C3b and enhanced cofactor activity in the Factor I-mediated cleavage of fluid phase and surface bound C3b (Tortajada et al. 2009). However in the structural model of the co crystal generated with the N-terminal region of Factor H and C3b, residue 62 in SCR 1 of Factor H is positioned outside the interaction interface of the two proteins (Wu et al. 2009).

3.1.2 Complement Factor H Related Proteins

Deletion of an 84-kbp fragment in the human Factor H gene cluster, which is positioned directly downstream of the Factor H gene has a protective effect for AMD development. The homozygous deletion of the chromosomal fragment results in the complete absence of the two plasma proteins **CFHR1** and **CFHR3** (Zipfel et al. 2007). CFHR1 is a regulator of both the C5 convertase and of the terminal pathway and thus controls reactions downstream of the C3 convertase, which is controlled by Factor H (Heinen et al. 2009). A CFHR1 related function is proposed for the CFHR3 protein (Hellwage et al. 1999).

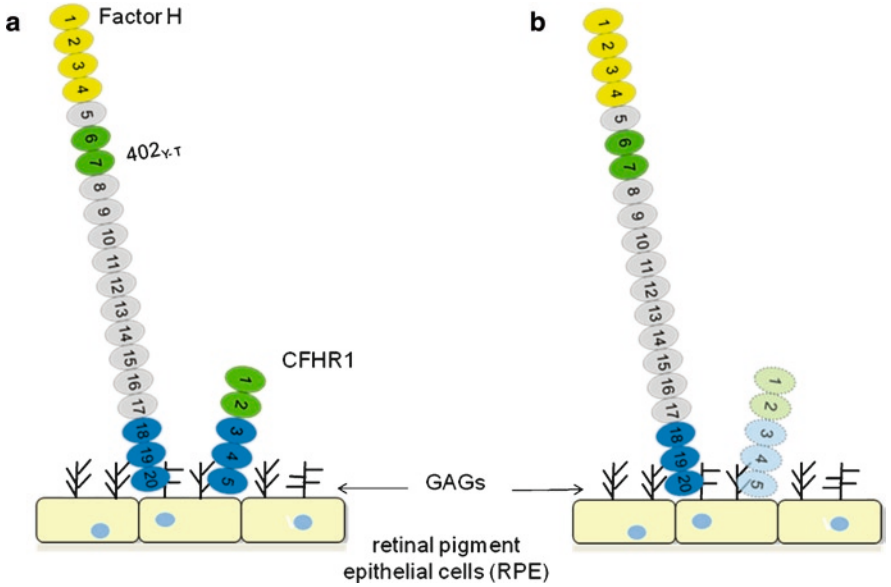

Fig. 2 Factor H and CFHR1 have identical C-terminal surface binding regions and bind to the same sites on the cell surface. (**a**) Factor H is composed of 20 SCR domains and CFHR1 by five domains. The three C-terminal SCRs of Factor H and that of CFHR1 (shown in *blue color*) show almost sequence identity and bind to the same ligands on the cell surface. Both proteins are present in plasma and in vitreous fluid and bind simultaneously to the same sites and surfaces. The relevant amino acid exchange at position 402 of Factor H is in domain 7 and is indicated in the Factor H model. (**b**) Genetic data show that chromosomal deletion of the *CFHR1* gene, which results in the absence of CFHR1 in vitreous fluid and in plasma has a protective effect in AMD. In the absence of CFHR1 more binding sites for Factor H are available on the surface of retinal pigment epithelial cells, resulting in increased Factor H binding and in a stronger local protection

CFHR1 and Factor H share almost identical C-termini. The three C-terminal domains of CFHR1 (i.e. SCRs 3–5) and that of Factor H (i.e. SCRs 18–20) show sequence identity of >98% (Skerka et al. 1991). The C-terminal region of Factor H is central for surface recognition and cell binding and this activity is shared by CFHR1 (Jozsi and Zipfel 2008). In consequence CFHR1 and Factor H bind simultaneously to the same sites on the cell surface. In the situation of CFHR1 deficiency more binding sites for Factor H are available and accessible (Heinen et al. 2009) (Fig. 2).

3.1.3 Other Complement Proteins Associated with AMD: C2, Factor B and C3

Polymorphisms in complement component **C2** (E318D) and **Factor B** (L9H, R32Q) are protective for AMD development (Gold et al. 2006). C2 is a component of the classical complement pathway, and Factor B is involved in alternative pathway

activation. C2 as well as Factor B are expressed in the neural retina, RPE and choroid. Factor B is also identified in ocular drusen. Genetic and functional data suggest that the protective effect is most likely mediated by mutations in the Factor B gene than mutations in the C2 gene. While the AMD associated C2 variants are either conservative change or an intronic SNP, the Q32 variant of Factor B has reduced hemolytic activity (Lokki and Koskimies 1991). Furthermore is the L9H exchange located within the signal peptide and may affect secretion and processing of Factor B. The AMD associated Factor B variants modulate activation of the alternative complement pathway and therefore may lead to an overall complement deregulation.

The AMD-associated polymorphism in **C3** results in exchange of arginine (R) to glycine (G) at amino acid position 102 (Yates et al. 2007). This exchange affects the mobility of the protein and results in a "fast" C3F (fast) and a "slow variant" C3s (slow) (Botto et al. 1990). C3F, the risk variant for AMD, is also associated with renal disease including IgA nephropathy, as well as MPGN-II (Wyatt et al. 1987; Abrera-Abeleda et al. 2006) and has been reported to influence long-term success of renal transplants (Brown et al. 2006). R102 forms together with neighbored or adjacent amino acid residues a positively charged spot on the surface of the C3 molecule, which in C3b is in close proximity with negatively charged amino acids close to the thioester-containing domain (Janssen et al. 2006; Yates et al. 2007). Substitution of positively charged R102 to neutral G102 likely weakens the interaction between charged surfaces and potentially influences thioester activity. Therefore the AMD associated risk variant G102 may affect C3b-Factor H complex formation resulting in less efficient C3b inactivation on (retinal) surfaces. Consequently C3 convertase action is increased and more activations products in form of C3b, iC3b, C3d are deposited onto ocular cell surfaces and generate more anaphylatoxin C3a (Johnson et al. 2001; Nozaki et al. 2006).

3.2 Gene Products of the Chromosome 10q26: ARMS-2 and HRTA1

Chromosome 10q26 encodes two genes that are not related to complement and that are strongly associated with risk for AMD development. The AMD-associated polymorphisms of **ARMS2** (rs10490924; A69S) and **HTRA1** (rs11200638, promoter polymorphism) are in strong linkage disequilibrium that their possible effects are indistinguishable in statistical analysis (Jakobsdottir et al. 2005). Apparently the SNP in the ARMS2 genes shows stronger association with AMD than the HTRA1 gene. This may explain the high association of chromosome 10q26 with AMD (Kanda et al. 2007).

The ARMS gene has an open reading frame which encodes an evolutionary new protein of 12 kDa with so far unknown biological function. A deletion–insertion polymorphism resulting in loss of the polyadenylation signal in ARMS2 RNA is strongly associated with AMD and directly affects transcript formation. Consequently in homozygous individuals no ARMS mRNA is detected. In individuals with the

protective A69 variant the protein is expressed in the retina, placenta and weakly also in the kidney, lung and heart. The exact localization of AMRS2 is still not defined. Different studies report either a cytosolic or mitochondrial localization within retinal cells (Kanda et al. 2007; Fritsche et al. 2008; Wang et al. 2009). The ARMS2 protein seems associated with microtubules of the cytoskeletion and with mitochondrial outer membranes. However independent of the exact cellular location of ARMS2, the S69 variant seems not expressed and the absence of this protein leads to progression of AMD. Thus, identification of the functional biological role of ARMS2 will provide new insights in AMD development.

The HTRA1 gene on chromosome 10q26 encodes a serine protease that is expressed in murine and human RPE cells (Oka et al. 2004). HTRA1 likely regulates degradation of extracellular matrix proteoglycans. This activity can facilitate access of other degradative matrix enzymes, such as collagenases and matrix metalloproteinases, to their substrates (Grau et al. 2006). The SNP rs11200638 within the promoter region of the HTRA1 gene is strongly associated with development of CNV. Apparently the risk variation affects expression levels of the protein and in eyes of donors, homozygous for the risk variant RPE show increased expression by factor of 1.7, as compared to the protective variant (Dewan et al. 2006; Yang et al. 2006). Such higher HTRA1 protein levels may alter the integrity of Bruch's membrane, favoring invasion of choroid capillaries across the extracellular matrix, as described for the wet form of AMD. Together, these findings suggest a potential new mechanism for AMD pathogenesis, independent of the complement-mediated AMD progression.

4 Lessons Learned from Rare Disorders (HUS, MPGN)

Defective and inappropriate complement regulation can cause AMD. In addition to this frequent retinal disease AMD, two rare kidney disorders, in from of atypical Hemolytic Uremic Syndrome (aHUS) and Membrano Proliferative Glomerulo-Nephritis (MPGN II, also termed dense deposit disease) are also associated with Factor H gene mutations, CFHR1/CFHR3 deficiency as well as mutations/polymorphisms in genes coding for complement regulators C3, Factor B and Factor I (Thurman and Holers 2006; Zipfel et al. 2006). Thus related genetic defects suggest similar or highly related pathophysiological mechanisms. Thus defective local complement activation results in related local damaged, which however manifest in different organs (Zipfel 2009).

Gene mutations have been reported for the rare renal disorders aHUS (Perez-Caballero et al. 2001) and MPGN II (Appel et al. 2005). Very related mechanisms can affect the endothelial lining of the glomerulus (aHUS) or the glomerular basement membrane, as well as the retinal cell and surface layer the Bruch's membrane. This similarity suggests that the fine tuned, coordinated action of multiple complement components is essential to maintain the delicate balance of the complement system and to prevent inflammation and cell damage. An exchange of one single residue

in the DNA, RNA or amino acid sequence can already affect protein expression and protein function. Such an exchange can affect the coordinated action and the progression of the cascade and the delicate balance between activation and inhibition of the complement cascade (Skerka and Zipfel 2008).

The knowledge how gene defects in complement components are translated into local defective complement regulation allowed defining appropriate therapy for these rare disorders aHUS and MPGN II. The related pathomechanisms of AMD, atypical HUS and MPGN II is relevant to design and use complement and other anti-inflammatory agents to locally control complement activation in these different organs.

5 Outlook

Based on genetic, functional, and immunohistological data evidence has accumulated that AMD is caused by deregulated local complement activation which develops into chronic inflammation. As related mechanisms of defective complement control results in damage of renal tissues a common link between diseases is emerging which were initially considered unrelated disorders. This allows prediction of additional disease associated genes and allows defining novel targets for complement inhibition.

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Chapter 3
Multiple Interactions of Complement Factor H with Its Ligands in Solution: A Progress Report

Stephen J. Perkins, Ruodan Nan, Azubuike I. Okemefuna, Keying Li, Sanaullah Khan, and Ami Miller

Abstract Factor H (FH) is the major regulator of the central complement protein C3b in the alternative pathway of complement activation, and is comprised of 20 SCR domains. A FH Tyr402His polymorphism in SCR-7 is associated with age-related macular degeneration (AMD) and leads to deposition of complement in drusen. The unravelling of how FH interacts with five major physiological and patho-physiological ligands is complicated by the weak nature of these interactions, coupled with the multivalency of FH. Using multiple biophysical methods, we summarise our recent results for these five FH ligands: (1) FH by itself shows a folded-back SCR domain structure in solution, and self-associates in a manner dependent on electrostatic forces. (2) FH activity is inhibited by zinc, which causes FH to aggregate. The onset of FH-zinc aggregation for zinc concentrations above 20 μM appears to be enhanced with the His402 allotype, and may be relevant to AMD. (3) The FH and C-reactive protein (CRP) interaction has been controversial; however our new work resolves earlier discrepancies. The FH-CRP interaction is only observed when native CRP is at high acute-phase concentration levels, and CRP binds weakly to the His402 FH allotype to suggest a molecular mechanism that leads to AMD. (4) Heparin is an analogue of the polyanionic host cell surface, and FH forms higher oligomers with larger heparin fragments, suggesting a mechanism for more effective FH regulation. (5) The interaction of C3b with FH also depends on buffer, and FH forms multimers with the C3d fragment of C3b. This FH-C3d interaction at high FH concentration may also facilitate complement regulation. Overall, our results to date suggest that the FH interactions involving zinc and native CRP have the closest relevance for explaining the onset of AMD.

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1 Complement Factor H

Complement Factor H (FH) is a comparatively abundant serum protein, being found in concentration ranges of 0.235–0.81 mg/ml (Saunders et al. 2006). Complement itself comprises a set of 30–40 proteins that forms the basis for innate immunity (Walport 2001; Janeway et al. 2005). It is a major defence and clearance system in blood, being activated by pathogens such as bacteria by one of three pathways, the classical, lectin or alternative pathways. All three pathways lead to the activation of C3, the central complement component, to C3b. C3b formation ultimately leads to the formation of a membrane attack complex that lyses bacteria. Complement activation requires regulation, because too much C3b activation will damage the human host, while too little C3b activation means that the host becomes susceptible to infection and other forms of pathogenic attack. FH regulates C3b in the alternative pathway. Thus FH competes with the Factor B protease that binds to C3b to form the C3b convertase enzyme, FH accelerates the decay of this C3 convertase enzyme that produces C3b, and FH acts as a cofactor of the protease Factor I for the proteolytic cleavage of C3b to form the fragments C3d and C3c. FH activity is inhibited by transition metals such as zinc, which causes FH to aggregate strongly (Nan et al. 2008b). CRP binds to the lipids of damaged host cell surfaces. While earlier literature has been complicated by the use of denatured CRP in binding studies to FH, FH was recently shown to bind to CRP when the latter is present at high concentrations in solution (Okemefuna et al. 2010b). FH also binds to anionic polysaccharides that cover the surfaces of host cells but which are absent from most pathogenic bacteria, and heparin is a widely-used analogue of these polyanions. The functionality of FH thus involves a complex set of multiple interactions with physiological and pathophysiological ligands that include zinc, CRP, heparin and C3b.

A comprehensive understanding of FH-ligand interactions is difficult to achieve for several reasons. Analyses are complicated by the weak binding of FH to ligands with K_D values that correspond to moderate μM affinities, i.e. there are no strong affinities with K_D values in the nM range.[1] These μM affinities reflect the abundant μM concentrations of FH and C3 in serum, and mean that the key interactions involving FH involve only partially-formed complexes. Such weak interactions may be missed in biochemical assays, and this will be discussed below in relation to CRP. In addition, FH undergoes multivalent interactions with its ligands, and this includes FH self-association with itself. Multivalency and self-association means that the conventional analyses of protein complexes in terms of simple 1:1 interactions is often not adequate when studying FH-ligand interactions. As a third complication, many FH-ligand interactions exhibit a salt dependence because they are formed through opposing ionic interactions between FH and its ligands. To improve their affinities, buffer compositions with reduced salt levels of 50 mM NaCl have been used in place

[1] FH and ligand concentrations are reported in both mg/ml and μM units in this review. Units of μM permit comparison with dissociation constant K_D values reported in μM units. For a 1:1 FH-ligand equilibrium, the K_D value is given by [FH][ligand]/[complex], and K_D corresponds to the FH concentration at which the complex is 50% dissociated.

of the serum level of 137 mM NaCl. Below, it is shown that the use of 50 mM NaCl is a frequent promoter of non-physiological interactions between FH and its ligands.

The abundance of FH in serum is reflected by its involvement in disease. Age-related macular degeneration (AMD) is a common disorder that causes loss of central vision and is the leading cause of blindness in the elderly in the Western world. Another FH-associated disease is atypical haemolytic uraemic syndrome, which is a common cause of renal failure in young children, and many FH mutations giving rise to this are clustered towards the C-terminus of FH (Saunders et al. 2007). Renal aspects will not be discussed further in this review. A key feature of AMD is the presence of drusen which are extra-cellular deposits between the retinal pigment epithelium and the choroid in Bruch's membrane. Drusen are composed of over 200 proteins, including the complement proteins, in association with cross-linked lipids, and are formed between the Bruch's membrane and the retinal pigment epithelium (RPE). A common Tyr402His polymorphism in FH is associated with many AMD cases, this being the first genetic correlation to be identified between any protein and AMD (Hageman et al. 2005; Klein et al. 2005; Edwards et al. 2005; Haines et al. 2005). The accumulation of drusen-associated molecules with FH suggests an unregulated complement attack that leads to AMD. However an understanding of a molecular mechanism for FH in AMD is still being developed. This progress report summarises what is currently known about possible mechanisms involving the known FH ligands, where the potential roles of zinc and CRP have recently attracted much interest. Any investigation of molecular mechanisms is further disadvantaged by the nature of AMD, given that AMD is a slow disease that only becomes apparent after a life-span of about 50 years. Explanations for AMD in terms of FH must therefore take into account its slow progression, and this fits the scenario of the weak but significant interactions that involve abundant complement proteins.

2 Structure of Factor H

FH is composed of 20 short complement regulator (SCR) domains, each of length about 61 residues, joined by linkers of lengths between three and eight residues (Fig. 1a). Domains are small independently-folded protein subunits, while the linkers possess less structure and potentially permit conformational movement between the SCR domains. The SCR domains constitute the most abundant domain type in the complement system; other common names for these domains are "short consensus repeat" or "complement control protein". FH has N-terminal and C-terminal binding sites for C3b at SCR-1/4 and SCR-19/20 (Schmidt et al. 2008). There are two CRP binding sites within SCR-6/8 and SCR-16/20 of FH (see below: Okemefuna et al. 2010b). There are also at least two anionic binding sites for heparin at SCR-7 and SCR-20 of FH (Schmidt et al. 2008). Our homology models for the 20 SCR domains in FH provided many of the first structural explanations for these polymorphisms and mutations (Saunders et al. 2006, 2007). Inspection of these

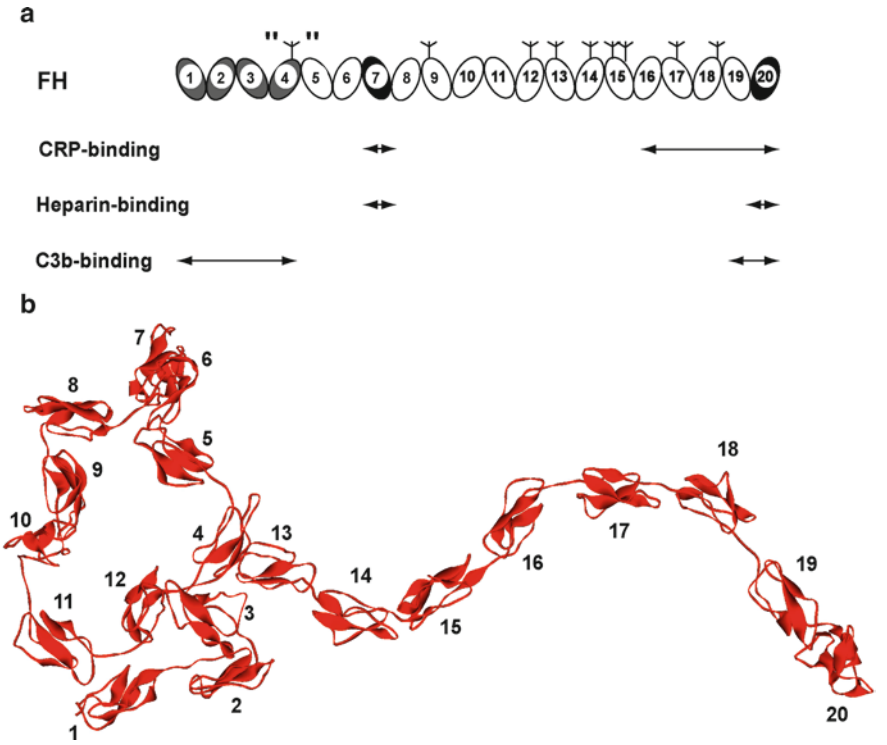

Fig. 1 Domain structure of FH. (**a**) Schematic cartoon of the 20 SCR domains of FH. The positions of nine putative N-linked glycosylation sites are shown, of which that in SCR-4 is not occupied (Fenaille et al. 2007). Major binding sites for CRP, heparin and C3b binding sites are indicated beneath the cartoon (Schmidt et al. 2008; Okemefuna et al. 2010b). (**b**) A molecular model of the folded-back domain structure for FH, in which the 20 SCR domains are numbered from 1 to 20. No specific proximity arrangement between non-adjacent SCR domains in FH is implied by this view, only that the SCR domains are generally folded back (Okemefuna et al. 2009a)

models on our interactive website http://www.fh-hus.org shows that the AMD-risk polymorphism Tyr402His is located on the surface of the SCR-7 domain. Another AMD-risk polymorphism Val62Ile is also located on the surface of the SCR-1 domain. These locations indicate that the polymorphisms play a role in the FH interactions with its ligands. These explanations were supported by the more detailed crystal or NMR structures that followed the homology modellings (Jokiranta et al. 2006; Prosser et al. 2007; Hocking et al. 2008; Wu et al. 2009).

To date, it has not yet been possible to crystallise intact FH. The absence of a high resolution FH crystal structure at atomic detail is attributable to its large size, glycosylation and inter-SCR flexibility. Instead, a solution structure for intact FH at medium structural resolution was determined by a combination of constrained scattering modelling and analytical ultracentrifugation (Aslam and Perkins 2001; Okemefuna et al. 2009a). The FH domain structure is not fully extended in solution, being partially folded-back (Fig. 1b). FH has a maximum length as low as 32 nm

in 137 mM NaCl buffer (Nan et al. 2008a). This is much reduced compared to the maximum possible overall length of about 73 nm if all the SCR domains are arranged in a straight line. Evidence for folding-back also comes from electron microscopy by others (DiScipio 1992). The SCR-1/5, SCR-6/8 and SCR-16/20 fragments also showed this partial folding-back (Fernando et al. 2007; Okemefuna et al. 2008). Both analytical ultracentrifugation and solution scattering also showed that the overall FH conformation is pH- and salt-dependent (Okemefuna et al. 2009a). Decreases in the FH sedimentation coefficient with increase in NaCl concentration showed that weak electrostatic interactions between different SCR domains caused its folded-back structure to become more compact. In addition, FH became more elongated at pH 9.4, showing that charged histidine residue(s) influence its folded-back structure. Thus the inter-SCR domain contacts in FH were promoted through the use of 50 mM NaCl buffer compared in 250 mM NaCl buffer. These medium resolution studies have been complemented by high resolution crystal and NMR structures for 11 of its 20 domains through the study of small two- and three-domain fragments by 2009 (Jokiranta et al. 2006; Prosser et al. 2007; Hocking et al. 2008). The high resolution studies show that the linker conformation between two adjacent SCR domains is not easily predicted, these orientations being variable between different domain pairs.

An understanding of the interactions of FH with its physiological ligands requires a molecular explanation of its folded-back conformation and the effect of ligand binding on this. Given that FH binds multivalently to several ligands with moderate μM affinities, it is thus necessary to characterise binding affinities to FH and correlate these with their physiological ligand concentrations. Below, five types of physiologically-relevant FH-ligand interactions will be summarised and their potential correlation with AMD will be discussed: (1) FH self-association; (2) FH interactions with transition metals including zinc; (3) FH interactions with CRP; (4) FH interactions with heparin; and (5) FH interactions with C3b and the C3d fragment of C3b. In addition, the potential importance of ternary or higher levels of FH-ligand complexes will be considered.

3 Self-Association of Factor H

The self-association property of FH was first clearly identified in 1991 where scattering studies of FH at comparatively high concentrations of 2 mg/ml (13 μM)[2] showed that its molecular weight was almost doubled (Perkins et al. 1991).

[2] There are literature discrepancies in the determination of FH concentrations. Recent mass spectrometry shows that eight of the nine putative N-glycosylation sites are occupied (Fenaille et al. 2007). From this and the FH sequence, the FH molecular mass is calculated as 154.4 kDa and the FH absorption coefficient at 280 nm (1%, 1 cm path length) is revised to be 16.2. Other groups reported a high absorption coefficient of 19.5 (Hakobyan et al. 2008a) or lower ones of 14.2 and 12.4 (Sim and DiScipio 1982; Pangburn et al. 2009). The value of 16.2 used in our work is conveniently close to the mean of these three experimental determinations.

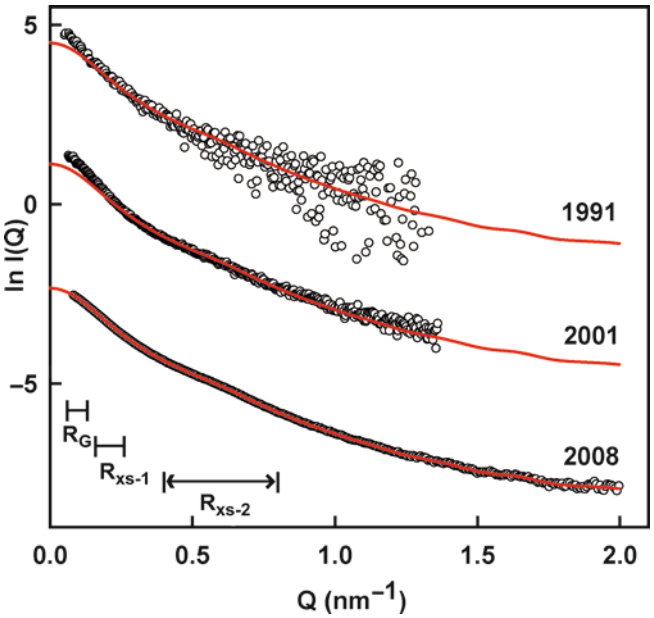

Fig. 2 Monomeric and oligomeric FH by X-ray scattering. X-ray scattering curves for FH reported previously are compared with each other (Perkins et al. 1991; Aslam and Perkins 2001; Nan et al. 2008a). FH concentrations were 2.0 mg/ml in 1991 and 3.6 mg/ml in 2001, both measured at the Daresbury synchrotron, and 4.3 mg/ml at the ESRF synchrotron in 2008. The continuous line corresponds to the fitted curve from 2008, which is then compared with the 1991 and 2001 curves. The Q ranges used to report the R_G, R_{XS-1} and R_{XS-2} values from the scattering data are indicated as *arrowed*

This was shown by the increased I(Q) intensities of scattering at the lowest scattering angle Q values in 1991 (Fig. 2). This result became controversial, when DiScipio (1992) argued from electron microscopy data that FH was monomeric, and our 2001 study at lower FH concentrations also suggested that FH was largely monomeric (Aslam and Perkins 2001). The controversy over the existence of FH dimers was eventually clarified when scattering and ultracentrifugation experiments with the FH SCR-6/8 fragment revealed the unambiguous existence of a monomer-dimer equilibrium with a measurable dissociation constant K_D of 40 μM (Fernando et al. 2007). The FH SCR-16/20 fragment also exhibited a stronger but similar self-association, with a monomer-dimer equilibrium K_D of 16 μM (Okemefuna et al. 2008). The existence of at least two different self-dimerisation sites at SCR-6/8 and SCR-16/20 resulted in the prediction that the presence of at least two dimer sites would lead to the formation of indefinite FH oligomers through the daisy-chaining of these dimer sites. The presence of FH dimers and smaller amounts of larger species ranging up to heptamers as well as monomers were confirmed by size-distribution analyses c(s) of ultracentrifugation sedimentation velocity experiments. A series of peaks 2–9 in the c(s) plot were seen (Fig. 3e) and these increased as the FH concentration increased (Nan et al. 2008a). The molecular modelling of plausible FH oligomer structures supported the peak assignment by verifying the sedimentation

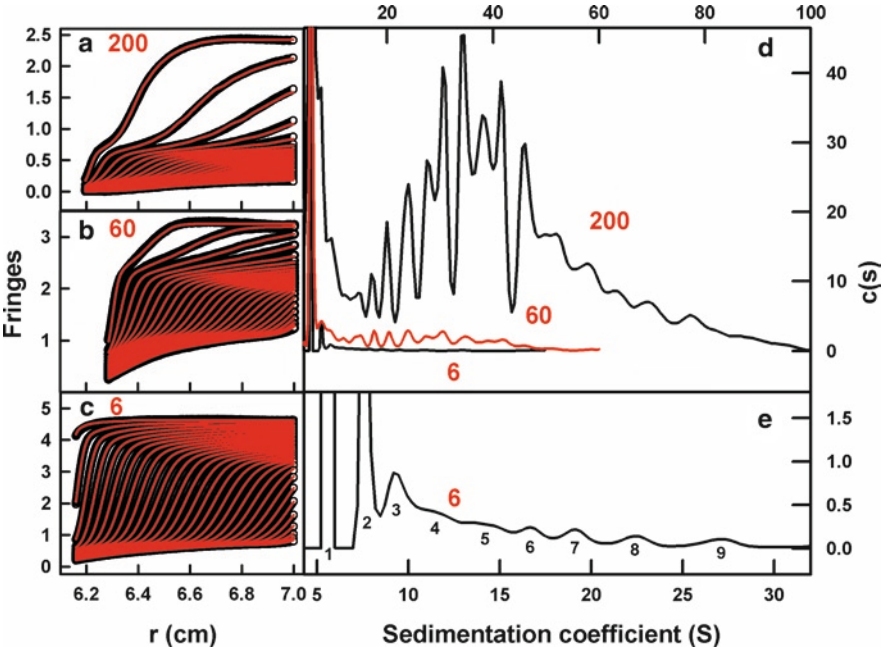

Fig. 3 Zinc-induced oligomerisation of FH. Ultracentrifugation velocity experiments of native FH at 0.87 mg/ml titrated with zinc at concentrations of 6, 60 and 200 μM (*red numbers*). (**a**–**c**) The experimental data are shown in *black* and the sedimentation boundary fits are shown in *red*. As the zinc concentration rises, the sedimentation rates become pronounced. (**d**) The *c(s)* size-distribution analyses for 6, 60 and 200 μM zinc are shown up to 100 S. The FH monomer peak occurs at 5.57 S. (**e**) The *c(s)* size-distribution analyses for 6 μM up to 30 S is expanded to show the FH oligomers (numbered 2–9) more clearly prior to zinc-induced aggregation

coefficients of these peaks 2–9. At low concentrations below 1 mg/ml, FH existed as a reversible monomer-dimer equilibrium with a K_D of 28 μM. At higher FH concentrations, oligomer formation was not reversible.

The FH dimers observed in 1991 were explained by the use of high FH concentrations required at that time for X-ray scattering. These high concentrations promoted oligomers. The FH monomers observed in 2001 were explained by the use of improved protein preparations, although increased I(Q) intensities were still seen at low Q (Fig. 2). The improved performance of the X-ray scattering equipment in 2008 meant that oligomer formation was much reduced (Fig. 2). In typical serum concentrations of FH, about 5–15% of FH will exist as dimer if no other factors are involved that will influence this equilibrium. Variations of the NaCl concentration and pH showed that FH oligomers prevailed in a wide range of buffer conditions (Okemefuna et al. 2009a). Thus FH self-association is an integral property of FH in vitro. The proper interpretation of FH-ligand experiments requires an allowance of FH self-association. Even though this property of FH has been known since 1991, FH dimerisation and its functional significance is often not discussed in high resolution structural studies (Prosser et al. 2007; Hocking et al. 2008; Wu et al. 2009) or in FH binding studies (Schmidt et al. 2008).

The question of whether FH dimer formation is relevant for AMD was evaluated by study of the SCR-6/8 fragment with Tyr402 or His402 (Fernando et al. 2007). A small propensity for the His402 allotype to form more dimer than the Tyr402 allotype was identified. At the level of intact FH, size-exclusion gel filtration and analytical ultracentrifugation do not show a significant difference between the FH His402 and Tyr402 allotypes (Nan et al. 2009). If homozygous FH His402 self-associates slightly more that homozygous FH Tyr402, this may contribute more to the slow formation of sub-RPE deposits during a lifetime.

4 Interaction of Factor H with Zinc

In addition to the Tyr402His and Val62Ile polymorphisms in FH that are associated with AMD, the accumulation of high zinc concentrations in the outer retina has also been associated with AMD (Lengyel et al. 2007). By the use of microprobe synchrotron X-ray fluorescence studies and fluorescent zinc-labelling studies, zinc concentrations of as high as 200 ppm (3 mM) have been identified in drusen. Interestingly FH function is inhibited by zinc, which causes FH to aggregate (Perkins et al. 1991). To investigate whether these two observations are linked, zinc-induced FH self-association was quantitatively studied by X-ray scattering and analytical ultracentrifugation alongside FH activity assays (Nan et al. 2008b). Titrations of pooled native FH at 0.42–1.05 mg/ml (2.8–7.0 μM) with zinc by X-ray scattering show that the scattering profile of FH was unaffected until [Zn] increased to 20 μM. Above this zinc concentration, the radius of gyration R_G values increased strongly from 9 nm up to 15 nm at [Zn] = 200 μM, indicating that the weak interaction of zinc with FH triggered the strong aggregation of FH. The maximum dimension of FH increased from 32 to 50 nm, indicating that the FH aggregates formed compact oligomers. The results were confirmed by analytical ultracentrifugation. There, size-distribution c(s) analyses showed that monomeric FH that sedimented at 5.57 S was the major species at [Zn] up to 60 μM (Fig. 3c). At [Zn] above 60 μM, a series of large oligomers were formed with much higher sedimentation coefficients ranging up to 100 S in size (Fig. 3a, b, d). FH-zinc oligomerisation was reversed by EDTA. Other transition metals were tested. Structurally distinct large FH oligomers were observed for Cu, while Ni, Cd and Fe showed lower amounts of FH oligomers, and Mg and Ca showed no effects. Fluid-phase assays with zinc and copper showed that the reduction of FH activities correlated well with the onset of oligomer formation. All these results were attributed to different degrees of stabilisation of weak self-dimerisation sites in FH by transition metals, of which zinc has the most pronounced effects.

In order to examine whether FH-zinc oligomer formation is relevant to AMD, zinc titrations were performed for the native Tyr402 and the AMD-risk His402 forms of FH (Nan et al. 2009). The AMD-risk allotype introduces a His residue which is a potential zinc-binding residue. The overall solution structures of both allotypes were indistinguishable by X-ray scattering and analytical ultracentrifugation.

In the presence of zinc, both allotypes strongly aggregated again at zinc concentrations above 20 μM. At present, while these investigations are not complete, our data suggest that the His402 allotype shows a slightly higher propensity to aggregate than the Tyr402 allotype in the presence of zinc (Nan et al. 2009). Given that the pathophysiological levels of zinc present in drusen are 200 ppm and higher, which is equivalent to about 3 mM zinc, these results have potential implications for the development of AMD.

The retina has the highest concentration of zinc per gram of tissue (Ugarte and Osborne 2001). It is possible that cellular damage to the RPE may release intracellular zinc into extracellular regions in the locality adjacent to Bruch's membrane where it comes into contact with FH. If locally-bioavailable zinc levels increase to above 60 μM, this would initiate oligomer formation at typical serum concentrations of FH. The resulting inhibition of FH through this mechanism might contribute towards the inflammation associated with AMD. In turn, this inflammation could release more zinc from RPE cells, and ultimately lead to the formation of sub-RPE deposits in the Bruch's membrane.

AMD is a progressive disease, and many years will elapse between the first appearance of sub-RPE deposits and the degeneration of the retina. Clinical trials indicate that zinc dietary supplements are able to slow the development of AMD in patients (AREDS research group 2001, 2002). The total zinc concentration in serum is 12.5 μM in serum, and remains low at 14.7 μM even after a daily supplement of 80 mg of zinc. Most of this zinc is tightly bound within serum proteins. This zinc level is well below the threshold of 20 μM zinc above which FH will aggregate with zinc. The improvements seen in AMD patients following zinc supplements are best explained if much retinal zinc is already trapped in the sub-RPE deposits, in which case further damage is reduced by enhancing zinc levels (Lengyel and Peto 2008). Interestingly, zinc supplements are effective in delaying the progression of AMD in patients who are homozygous for Tyr402, but not for His402 (Klein et al. 2008). This shows that the FH-zinc interaction is significant for the development of AMD, possibly through the propensity of FH His402 to aggregate more strongly with zinc than FH Tyr402.

5 Interaction of Factor H with C-Reactive Protein

CRP (molecular mass 115 kDa) is the classic acute phase protein. During the acute phase, its serum protein concentration increases rapidly by over 1,000-fold from a median level of 800 ng/ml in normal serum up to 0.5 mg/ml (from 7 nM to 4.4 μM). CRP is composed of five lectin-like subunits that forms a discoid arrangement that bind ligands in a Ca^{2+}-dependent manner. Note that 2 mM Ca^{2+} is present in serum (Hurwitz 1996). CRP binds to phosphorylcholine, as well as phosphoethanolamine, microbial surface proteins, chromatin and other ligands (Pepys and Hirschfield 2003; Black et al. 2004). Ca^{2+} and phosphorylcholine bind to the B face of the pentameric disc. These CRP-ligand interactions lead to the labelling of damaged or apoptotic cells and bacterial pathogens for recognition purposes. CRP activates the

classical pathway of complement by binding to C1q at the other A face of the pentamer (Thompson et al. 1999).

A FH interaction with CRP would make sense if complement activation through CRP requires regulation. The study of this FH-CRP interaction was complicated by five factors: (1, 2) the self-association of each of FH and CRP, (3) the susceptibility of CRP to denaturation, and (4, 5) the importance of protein concentrations and buffer composition. Prior to our re-investigation of the FH-CRP interaction (Okemefuna et al. 2010a, b), 9 out of 11 earlier studies had concluded that FH and CRP formed a complex, while two studies concluded that complex formation had only occurred because of the presence of denatured CRP. A critical evaluation of these earlier FH-CRP studies showed that the full physiological FH and CRP concentration range had not always been employed, low salt buffers with 50 mM NaCl and not 140 mM NaCl had sometimes been used, 2 mM Ca^{2+} had often been omitted from the buffer or was not specified, and two different levels of FH-CRP interactions had been observed with approximate K_D ranges of either about 1 μM or about 0.01 μM. We believe that these discrepancies in these 11 earlier FH-CRP studies have now been resolved by our recent studies (Okemefuna et al. 2010a, b).

An understanding of both CRP and FH oligomerisation proved essential to interpret the FH-CRP interaction. CRP self-association was already known from concentration studies by scattering and ultracentrifugation (Perkins and Pepys 1986; Blizniukov et al. 2003). Ultracentrifugation size-distribution analyses c(s) showed two species through the observation of peaks P and D for CRP, with a stable sedimentation coefficient of 6.4 S for the first peak, and a concentration-dependent one of above 7.6 S for the second peak (Fig. 4). The 6.4 S peak corresponds to pentameric CRP, while the 7.6 S peak was assigned to a fast equilibrium between pentameric and decameric CRP. By X-ray scattering, a pentamer-decamer K_D value of 21 μM in solution was determined from scattering curve fits based on crystal structures. CRP self-association was verified by surface plasmon resonance which showed that soluble CRP bound to immobilised CRP with a similar K_D value of 23 μM. Interestingly, CRP aggregated with immobilised CRP in a low salt buffer of 50 mM NaCl. Overall, it was deduced that CRP exists in a reversible pentamer-decamer equilibrium in a physiologically-relevant concentration range, and that a buffer with both 2 mM Ca^{2+} and 140 mM NaCl was essential for consistent results.

Next, experiments with CRP needed to avoid its denaturation into monomeric CRP (mCRP). This is formed slowly in the absence of 2 mM Ca^{2+}. Because Ca^{2+} binds to CRP with a moderate dissociation constant K_D of 30–60 μM, 2 mM Ca^{2+} is required for full occupancy of the CRP calcium binding sites and its stabilisation (Kinoshita et al. 1989; Christopeit et al. 2009). Ultracentrifugation showed that CRP partially dissociates into its protomers when insufficient Ca^{2+} is present. Compared to CRP, mCRP has different biological and antigenic properties, and shows a high affinity for FH. Because denatured CRP is normally rapidly catabolised and does not persist, mCRP is not considered to occur physiologically (Pepys and Hirschfield 2003). In 2007, five studies suggested that FH bound to immobilised CRP in ELISAs, and that the wild-type FH Tyr402 allotype bound more strongly to CRP than the AMD-risk His402 allotype (Herbert et al. 2007; Laine et al. 2007; Sjöberg et al. 2007; Skerka et al. 2007; Yu et al. 2007). Two other studies concluded

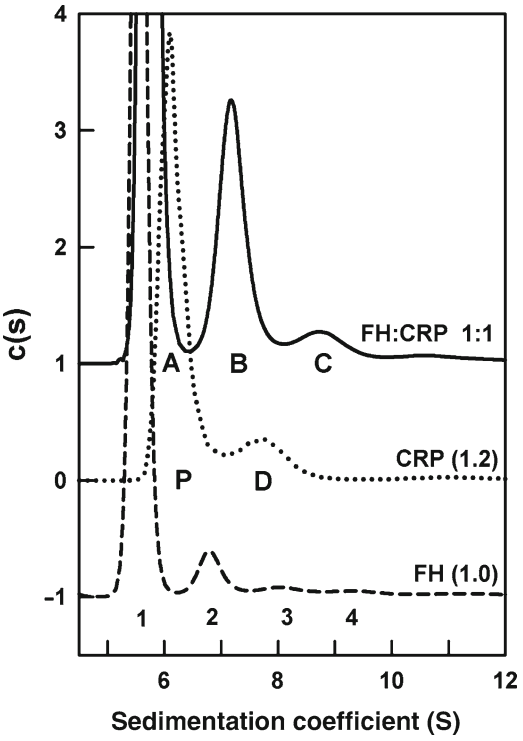

Fig. 4 Ultracentrifugation of FH, CRP and their complex. The *c(s)* size-distribution analyses showed that FH and CRP each formed oligomeric species, and these disappeared on mixing FH and CRP in buffers containing 2 mM Ca^{2+} and 140 mM NaCl. The data for FH at 1.0 mg/ml showed monomer (peak 1) and small amounts of dimer, trimer and tetramer (peaks 2–4) that are visible on the scale shown. The data for CRP at 1.2 mg/ml showed pentamer (P) and decamer (D). The 1:1 molar ratio mixture of FH and CRP showed that new peaks A, B and C formed at different S values from those of peaks 1, 2, 3, P and D, and the higher oligomers of FH disappeared (Okemefuna et al. 2010b)

that the FH-CRP interaction did not exist because of the inadvertent presence of mCRP in earlier studies that had preferentially bound to FH instead (Bíró et al. 2007; Hakobyan et al. 2008b). In 2008, the observed FH-CRP interaction seen in 2007 was attributed to the inadvertent denaturation of CRP by Ca^{2+} removal in surface plasmon resonance or the use of plastic-coated surfaces in ELISA assays which also denatures CRP (Hakobyan et al. 2008b).

To resolve the different claims for the existence of the FH-CRP interaction, FH and CRP were studied together both in solution and on surfaces as controls of self-consistency. By ultracentrifugation in 140 mM NaCl and 2 mM Ca^{2+}, CRP was unexpectedly seen to suppress oligomer formation of FH (Fig. 4). The size distribution c(s) plots showed the expected FH monomer and CRP pentamer peaks, together with the clear removal of the peaks for the higher FH oligomers. Further examination showed that the c(s) peaks for the FH dimer and CRP decamer were also diminished (Fig. 4). The same results were obtained using surface plasmon resonance using both

FH-immobilised or CRP-immobilised chips as controls, when binding was again observed. The two FH-CRP data sets showed that, using physiological FH and CRP concentrations in the appropriate buffer, FH-CRP binding occurred with a K_D of 4 μM (Okemefuna et al. 2010b). Note that binding to FH is only observed at high CRP concentrations that correspond to acute phase levels that arise after inflammation or infection, not at the resting concentration of CRP in serum.

The removal of the higher oligomers of FH after the addition of CRP indicated that the FH self-association sites at SCR-6/8 and SCR-16/20 were both blocked. This suggested that there were two different non-contiguous binding sites for CRP in FH. Experiments using surface plasmon resonance verified this, in which it was shown that each of SCR-6/8 and SCR-16/20 bound to immobilised CRP, but that SCR-1/5 did not bind (Okemefuna et al. 2010b). To investigate whether the Tyr402His AMD-risk polymorphism was relevant to CRP binding, both forms of

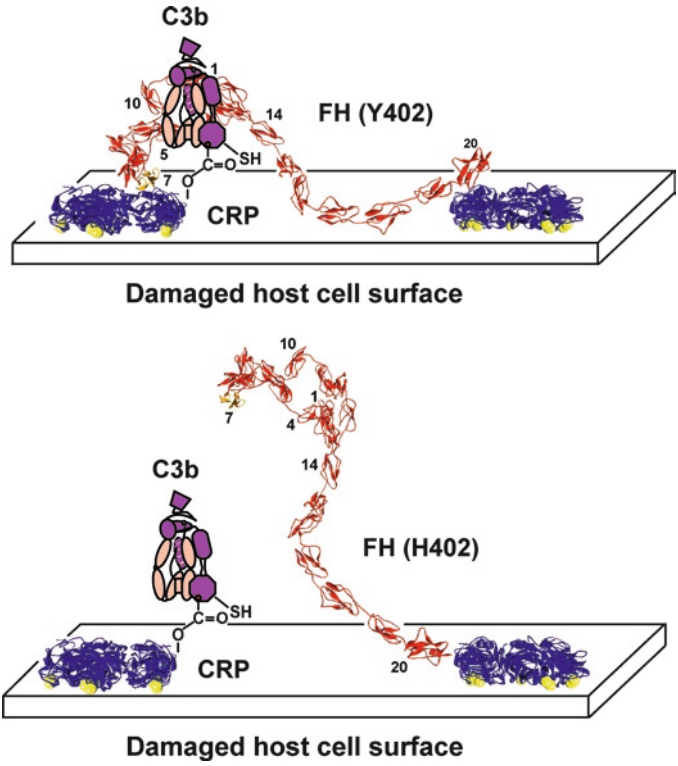

Fig. 5 Cartoon of a proposed FH-CRP mechanism that may lead to AMD. A schematic illustration of how CRP can recruit wild-type FH (Y402) at two binding sites onto CRP-coated surfaces on damaged host cells at both SCR-7 and SCR-20. This positions SCR-1/4 in an appropriate location to mediate the degradation of surface-bound C3b. This will limit complement activation at the host cell surface during inflammation. In contrast, FH (His402) is less able to regulate C3b degradation because of the weaker binding of SCR-7 (His402) to CRP. This is postulated to result in greater host cell damage (Okemefuna et al. 2010b)

SCR-6/8 were studied. A large difference in binding was seen between the wild-type allotype (Tyr402) and AMD-risk allotype (His402) SCR-6/8 binding to native CRP. The K_D values of 4 μM (Tyr402) and 12 μM (His402) showed a threefold change in affinity. This difference is larger than observed in the five 2007 studies.

In conclusion, the new investigations into the FH-CRP interaction clarified that this does in fact take place. The Okemefuna et al. (2010b) study was selected as a "Paper of the Week" by the editors at J. Biol. Chem. The difference seen between the Tyr402 and His402 allotypes of FH in binding to native CRP (and not denatured CRP) provides molecular insight into an AMD-causative mechanism (Fig. 5). In non-acute phase conditions, CRP serum concentrations will be low at 7 nM, and CRP and FH do not interact because the K_D is 4 μM. During the acute phase response, the increased serum levels of CRP at 4.4 μM are high enough to become important. Excessive complement inflammation is then brought under control in regions where CRP has decorated damaged host cells. This FH regulation becomes less effective for the His402 AMD-risk polymorphic form, which is threefold less able to bind to native CRP, and interacts less well with surface-bound C3b (Fig. 5). During the progression of a human lifespan, it can be seen that an accumulation of successive acute-phase reactions may increase the risk of AMD for individuals who are heterozygous or homozygous for FH His402. Interestingly, individuals who are homozygous for the AMD-risk His402 FH allotype show a 2.5-fold higher level of CRP in the RPE (Johnson et al. 2006). Presumably higher CRP levels are required in these individuals in order that CRP will interact with the FH His 402 allotype.

6 Interaction of Factor H with Heparin

FH regulates surface-bound C3b activity by recognising polyanionic structures such as sialic acid, heparan sulphate and dermatan sulphate on host cell surfaces and thereby inhibits complement activation on host cells. Heparin is often used as an analogue for these polyanions. Despite the knowledge of a NMR solution structure for unbound heparin and 19 crystal structures for heparin-protein complexes based on short heparin fragments, no structures for larger heparin fragments in solution have been reported until recently (Khan et al. 2010). Solution structures for six purified heparin fragments of sizes dp6 to dp36 (i.e. 3–18 iduronate and glucosamine disaccharide units) were determined by a combination of analytical ultracentrifugation, synchrotron X-ray scattering, and constrained modelling. The ultracentrifugation data showed sedimentation coefficients that increased linearly with heparin size. The corresponding X-ray scattering studies resulted in radii of gyration R_G and maximum lengths that also increased with heparin size. The higher structural resolution of X-ray scattering showed that the heparin solution structures became progressively more bent with increased size. The computerised molecular modelling of randomised heparin conformers revealed best-fit structures for dp18, dp24, dp30 and dp36 that indicated moderate flexibility in mildly bent structures (Fig. 6). The comparison of these

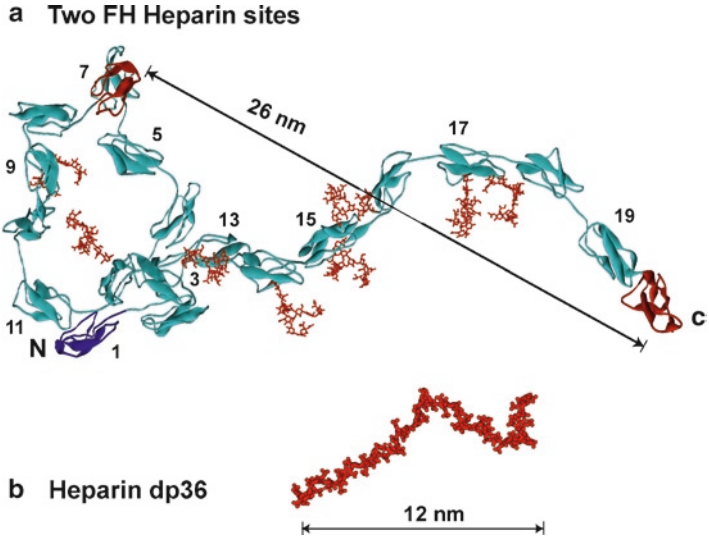

Fig. 6 Structural comparison of FH and heparin dp36. The solution structures of SCR 1–20 in FH and heparin dp36 are compared with each other on the same scale. The heparin-binding domains at SCR-7 and SCR-20 are shown in *red*, together with eight N-linked oligosaccharide chains in *red*. SCR-1 is shown in *blue*. The separation of SCR-7 and SCR-20 is 26 nm. Heparin dp36 binds to FH to cause FH oligomerisation. Since dp36 is 12 nm in length, it is unlikely to bind simultaneously to both SCR-7 and SCR-20 within one FH molecule (Khan et al. 2010)

solution structures with the crystal structures of heparin-protein complexes revealed similar orientations between the iduronate and glucosamine rings both in the solution and the crystal states. It was concluded that heparin has a semi-rigid and extended conformation that is pre-formed and optimal for binding to protein targets such as FH without major conformational changes (Khan et al. 2010).

Heparin interacts with FH in at least two positions at SCR-7 and SCR-20 (Figs. 1a and 6). The FH SCR-6/8 fragment showed a bent SCR domain arrangement that binds heparin dp10 in a strong 1:1 complex in 137 mM NaCl buffer (Fernando et al. 2007).[3] Changes in the X-ray scattering curve on the addition of dp10 suggested either that SCR-6/8 changed conformation or formed oligomeric structures on binding to heparin. The Tyr402His AMD-risk polymorphism lies on

[3] The determination of the FH SCR-6/8 solution structure, its dimerisation and its interaction with heparin dp10 (Fernando et al. 2007) was not discussed in the subsequent high resolution crystal structure study of SCR-6/8 with the heparin analogue SOS (Prosser et al. 2007). Three authors of the second study were also co-authors of the first study. The two SCR-6/8 structures were very similar, but this was not acknowledged in the second study. The second study also suggested that heparin dp18 and dp24 formed an extended 1:1 complex with SCR-6/8. These further conclusions did not consider SCR-6/8 dimerisation or heparin-induced changes described in the first study. To date, corrections requested by the journal editor from the authors of the second study to clarify the relationship to the first study have not been published.

the surface of SCR-6/8 and may be involved with heparin binding. A SCR-6/8 crystal structure co-crystallised with SOS, a polysulphated monosaccharide analogue of heparin, suggested that the Tyr402His polymorphism was related to altered heparin binding (Prosser et al. 2007). As for CRP, different studies on the affinity of the SCR-6/8 region with heparin for the Tyr402 and His402 allotypes showed different outcomes. Three studies reported a higher affinity of heparin for the Tyr402 allotype compared to His402 (Herbert et al. 2007; Skerka et al. 2007; Ormsby et al. 2008). This outcome is consistent with that for the FH-CRP interaction (Okemefuna et al. 2010b) in that the weaker binding of FH His402 to host cell surfaces would predispose towards greater inflammatory damage. However a fourth study reported variable outcomes depending on the heparin preparation in use and its degree of sulphation (Clark et al. 2006), while a fifth study reported that no significant difference was observable between the Tyr402 and His402 allotypes (Yu et al. 2007).

Up to the solution structural determination of heparin, it had not been clear whether SCR-7 and SCR-20 could bind simultaneously to the same heparin oligosaccharide. The new dp18-dp36 solution structures and the solution structure for intact FH (Aslam and Perkins 2001; Okemefuna et al. 2009a; Khan et al. 2010) indicate that there is insufficient flexibility to facilitate the simultaneous binding of two heparin sites within one FH molecule to one heparin molecule. The lack of conformational flexibility makes it more likely that FH cross-links different heparin oligosaccharides to form very large oligomers, and these have been observed (Khan et al. 2008; Pangburn et al. 2009). This may indicate that at least two different heparin sites evolved within FH (Fig. 6) in order to facilitate the selective binding of FH to host cells that display large polyanionic surfaces, and not to pathogens that lack multiple polyanionic binding sites. In this sense, the FH-heparin interaction resembles those of the FH-CRP interactions (Fig. 5). A re-clarification of the contribution of the His402 polymorphism to heparin binding with FH is currently in progress.

7 Interaction of Factor H with C3d

Complement activation results in the conversion of C3, the so-named third component of complement, into its activated form C3b. At 1.0 mg/ml, C3 (190 kDa) is the most abundant complement component in serum, and its level can be considerably elevated during inflammation and infection. The C3 convertase enzyme complex cleaves C3 to form C3b and a small anaphylatoxin C3a. The removal of C3a induces a conformational change in C3 in which the TED domain within C3 (Fig. 7a) moves along C3 to expose an internal thiolester group in C3b, which is inaccessible in native C3 (Janssen et al. 2005). The thiolester forms a covalent bridge with nearby cell surfaces. When factor I cleaves C3b with FH as cofactor, cleavage takes place at two sites in C3b to release the TED domain (otherwise known as C3d) from the C3c fragment. The C3d fragment (35 kDa) contains the active site region of C3b, while C3c contains the regions required for convertase formation, thus their separation abrogates activity.

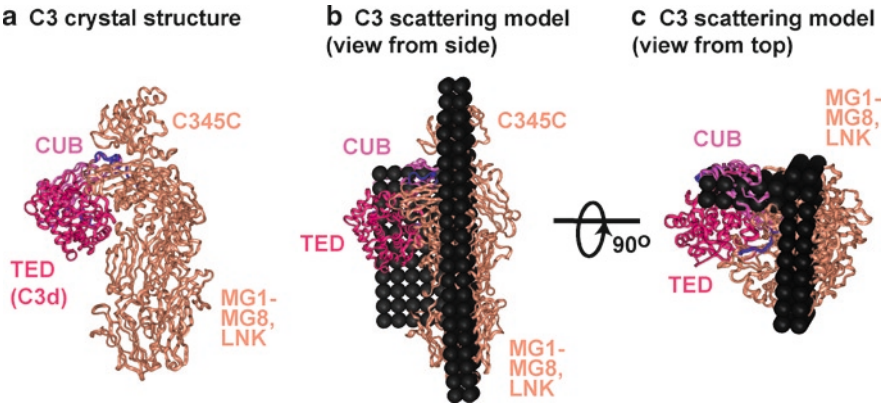

Fig. 7 Comparison of the C3 crystal and solution structures. The crystal structure of C3 is compared with the solution structure of C3 determined by X-ray and neutron scattering fits (Perkins and Sim 1986; Perkins et al. 1990; Janssen et al. 2005). The TED domain of C3 is equivalent to the C3d fragment, and possesses the reactive thioester group that becomes attached to cell surfaces. The TED domain is connected via the CUB domain to the remaining MG1–MG8 and LNK domains of C3. The protease Factor I cleaves the activated structure C3b with the assistance of FH as cofactor. Comparison with the low resolution solution structure of C3 (*black spheres*) show that the overall dimensions of the crystal and solution structures are in good agreement

High resolution crystal structures are known for C3, C3a, C3b, C3c and C3d and several of their macromolecular complexes, discussed elsewhere (Gros et al. 2008). The C3 crystal structure is similar to its low resolution solution structure determined from scattering modelling, both in terms of its dimensions and the distinct and separate location of the TED and CUB domains (Perkins and Sim 1986; Perkins et al. 1990) (Fig. 7b, c). The solution properties of these C3 proteins and their interactions with ligands such as FH are less well known.

FH binds in at least two different locations to C3b, with one site at SCR-1/4 and the other at SCR-19/20 (Schmidt et al. 2008). C3d binds at SCR-19/20. X-ray scattering of free C3d showed a pronounced concentration dependence in its R_G values that suggested an apparent monomer-dimer dissociation constant K_D of 23 ± 3 μM in 50 mM NaCl (Gilbert et al. 2005; Perkins and Furtado 2005). The C3d crystal structure gave good curve fits for monomers at low concentrations (Gilbert et al. 2005). However a reinvestigation by analytical ultracentrifugation revealed that this presumed equilibrium is better described as a monomer-dimer-trimer equilibrium (Li et al. 2008). In a physiological 137 mM NaCl buffer, C3d becomes monomeric. This again exemplifies the pitfalls of working in 50 mM NaCl. Even though low salt will magnify weak ionic interactions between proteins to make these observable, this can lead to non-physiological binding artefacts, as noted above for CRP.

Solution studies of the FH-C3d interactions revealed a complicated story. Given that each of FH and C3d formed multimers in various buffer conditions, a multidisciplinary strategy using both 50 mM NaCl and 137 mM NaCl buffers became necessary to decipher this (Okemefuna et al. 2009b). Several peaks were seen in size distribution c(s) plots and showed that multiple complexes were formed on

mixing C3d with either SCR-16/20 or FH, not the expected 1:1 complexes. The concentration dependence of the c(s) peaks showed that the equilibria were weak ones with comparatively rapid on/off rates. These observations were confirmed by surface plasmon resonance. The best binding fits showed that a simple 1:1 model was unable to account for the interaction, and rapid on/off rates were again observed. X-ray scattering also indicated that the data could not be explained in terms of a 1:1 interaction. Using molecular models, and starting from an assumed monomer-dimer equilibrium for each of SCR-16/20 and C3d, it was possible to show how C3d dimers and SCR-16/20 dimers could alternate with each other to account for the peaks seen in the c(s) analyses. Repeating these experiments with FH-C3d mixtures showed that similar multimerisation events also occurred.

The FH-C3d study provided interesting insight into complement regulation (Fig. 8). After cleavage of host-cell bound C3b, C3d is left bound to the host cell surface. In conditions of high C3 concentrations and amplified activation rates to form C3b, host cells will be significantly coated with C3d on their surfaces. These high local concentrations of C3d will promote more FH binding via the formation of multimeric FH-C3d complexes. This will increase the concentration of host-cell surface-bound FH to provide additional complement regulatory protection of host cells under chronic inflammatory conditions.

The CR2-C3d interaction illustrates a very different aspect from the FH-C3d interaction. CR2 with 15 SCR domains is a membrane glycoprotein found at cell surfaces of mature B lymphocytes and follicular dendritic cells. Functional studies show that both CR2 SCR-1 and SCR-2 bind to C3d. Two crystal structures for

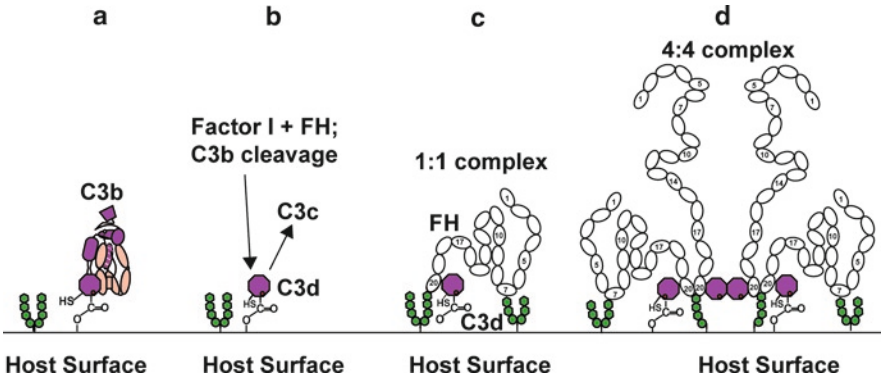

Fig. 8 Cartoon of a multimeric association of FH and C3d on host cell surfaces. Schematic cartoons of the multimeric complexes formed between FH and the C3d fragment at the host cell surface when high amounts of C3d and FH are present (Okemefuna et al. 2009b). (**a**) Activated C3b is covalently bound through its thioester group to the host cell surface. The host cell surface also presents anionic oligosaccharides (analogous to heparin). (**b**) FH degrades surface-bound C3b through factor I cleavage to leave C3d bound to the host cell surface, releasing C3c in the process. (**c**) FH forms a 1:1 complex with both host cell-bound C3d and with anionic oligosaccharides at SCR-20 and SCR-7. (**d**) A multimeric 4:4 complex of FH:C3d is shown. This suggests how high amounts of soluble and cell-bound C3d will lead to FH multimerisation with C3d on the host cell surface, thereby enhancing the host cell protection from excessive C3b

unbound SCR-1/2 and its complex with C3d showed that the two SCR domains formed a sharply folded-back V-shaped arrangement of SCR domains, and only SCR-2 made contact with C3d (Szakonyi et al. 2001; Prota et al. 2002). X-ray scattering fits in 50 mM NaCl showed that the linker peptide between SCR-1 and SCR-2 resulted in an opened-up V-shaped structure in solution (Gilbert et al. 2005). CR2 SCR-1/2 formed a strong 1:1 complex with C3d in 50 mM NaCl. Further X-ray scattering fits now showed that both SCR domains make contact with the surface of C3d in solution (Gilbert et al. 2005), and mutagenesis experiments with CR2 SCR-1/2 confirmed that SCR-1 interacted with C3d (Hannan et al. 2005). The same studies of complex formation between intact CR2 and C3d in 137 mM NaCl showed that this complex was not detectable (Li et al. 2008). This raises an interesting issue of how CR2 and C3d can interact physiologically in 137 mM NaCl. It was resolved by postulating that in vivo CR2 and C3d interact for reason of the increased local concentrations of proteins at cell surfaces (Li et al. 2008). Weak interactions will be amplified by large amounts of CR2 molecules on the B-cell surface and likewise on the C3d-coated pathogenic surface. In other words, unlike the FH interactions, this mechanism means that B-cells will respond specifically only to C3d-coated pathogens. i.e. CR2 on its own will not bind to unbound free C3d in serum.

8 Conclusions and Future Considerations

Complement is effective in attacking pathogens while not damaging the human host, and imbalances in this process are associated with disease conditions such as AMD. FH interacts with at least five distinct physiological or patho-physiological ligands, i.e. to itself, zinc, CRP, heparin and C3b. A biophysical review of the FH-CRP interaction is presented elsewhere (Perkins et al. 2010). Here, we have summarised our current views of these interactions, and explain their relevance for possible molecular mechanisms that lead to AMD. Either aggregation (deposit-forming) or inflammation processes may be involved (Table 1). The major new insight from recent work clarifies the importance of association and oligomeric events for FH function.

The unravelling of functional FH interactions requires a multi-disciplinary approach. Despite the power of crystallography (Gros et al. 2008), there are no all-embracing single “standalone” methods. Here, the joint application of four methods, namely synchrotron X-ray solution scattering, analytical ultracentrifugation, constrained molecular modelling and surface plasmon resonance have started to provide major clarifications of the FH interactions. While these methods are lower resolution techniques than crystallography, there is now a widespread recognition that in order to understand biological systems in a meaningful and integrated way, such as complement, it is necessary to apply several techniques that cover structures and interactions from atomic levels to cellular levels.

The complement complexes are often weakly formed, making experiments more difficult. Complement functions through a coordinated series of weak fluid-phase

Table 1 Current understanding of FH-ligand interactions in relation to AMD

Increased protein aggregation at retinal cell surface?

1. FH self-association means that up to 15% of FH circulates in plasma as dimer and higher oligomers. FH self-association may be relevant to drusen formation. *Low significance.*
2. FH His402 aggregates slightly more in the presence of zinc in the retina. This could provide a slow mechanism for deposit growth during a human lifetime. *Possibly significant.*
3. Acute-phase CRP binds to FH at two different sites and suppresses FH multimers. This will not contribute to aggregation and deposit formation. *Not significant for AMD.*
4. Heparin can cross-link FH molecules on host cell surfaces and may lead to deposit growth. Nothing specific with His402 allotype is known. *Do not know.*
5. FH-C3d multimers may lead to accumulation of protein at cell surfaces. Nothing specific with His402 allotype. *Not significant.*

Increased inflammation at retinal cell surface?

1. FH self-association may inhibit ability of FH to regulate C3b at cell surfaces. Nothing specific with His402 allotype. *Low significance.*
2. FH His402 may aggregate slightly more with zinc. This could block C3b binding and provide a slow mechanism to increase inflammation during a lifetime. *Possibly significant.*
3. Acute-phase levels of CRP binds weakly to FH His402, so this will reduce the level of C3b regulation at surfaces and lead to more inflammation. *Probably significant.*
4. The binding of FH to polyanionic heparin is weaker in most studies with the His402 allotype, implying weaker FH binding at host cell surfaces. *Possibly significant.*
5. FH-C3d multimers may promote protein accumulation at host cell surface during inflammation. Nothing specific with His402 allotype. *Not significant.*

binding events using comparatively abundant serum proteins. The complement proteins often interact through ionic interactions. These events are amplified (when required) through increased local concentrations at cell surfaces. In the case of FH, its multivalency for its ligands (Fig. 1) complicates experimental analyses, but multivalency is expected to contribute towards the effectiveness of FH. By these means, complement is targeted appropriately. Because complex formation is promoted in low salt conditions, the ionic strength of the buffer is commonly reduced in experiments from physiological (137 mM NaCl) to 50 mM. Unfortunately low salt conditions can lead to artefacts, and examples above have been reported for CRP, C3d and the CR2-C3d complex. Other experimental issues are exemplified by the FH-CRP complex. There, the protein concentrations, the inadvertent denaturation of CRP, and the requirement for calcium are additional factors for consideration.

A major insight from the solution and surface-phase studies of FH with its ligands is the common occurrence of association in FH and FH-ligand complexes. Native FH itself is principally monomeric and reversibly forms 5–15% of dimers in the concentrations seen in serum if no other factors are involved. Higher but less reversible FH oligomer formation occurs at high FH concentrations. Zinc promotes strong FH aggregate formation that is reversed by EDTA and zinc inhibits FH activity. Native CRP exists as pentamers and decamers at acute-phase concentrations of CRP. When mixed together, both native FH (Tyr402) and CRP suppress both these self-associations, whereupon FH forms bivalent complexes with CRP. When heparin is present, this also promotes FH oligomer formation. FH and C3d form a multimeric

series of oligomers. The frequent observations of FH-based oligomers are explained by the existence of multivalency in FH for the same ligand, suggesting that these oligomers are in fact important for function.

The current relevance of the five FH interactions for AMD is summarised (Table 1). FH alone may exhibit a slightly greater propensity for self-aggregation, indicated by the comparison of both allotypes of SCR-6/8 which indicated that the His402 aggregated more (Fernando et al. 2007). The addition of zinc to both allotypes of SCR-6/8 likewise may favour a slightly higher level of aggregation for the His402 form (Nan et al. 2009). Both events may facilitate a slightly enhanced but slow growth of drusen over a lifetime. A larger difference is seen between the two allotypes in the case of CRP binding to FH, and this is a more promising explanation for the onset of AMD that involves the His402 polymorphism (Okemefuna et al. 2010b). The two allotypes may differ in terms of their heparin binding properties, however this requires further investigation. It is possible that, in terms of FH, AMD may not necessarily reflect a single FH binding event, and several factors may be jointly relevant.

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Chapter 4
Genetic Control of Complement Activation in Humans and Age Related Macular Degeneration

Laura A. Hecker and Albert O. Edwards

Abstract The major focus of our research is to understand how age-related macular degeneration (AMD) develops. It is known that genetic variation can explain much of the risk of developing AMD. However, we do not know what controls the transition between a normal fundus and the extensive accumulation of subretinal inflammatory material that we recognize as drusen in AMD. We do know that the accumulation of this inflammatory material that characterizes the maculopathy underlying AMD is by far the most important predictor of late AMD. Late or advanced forms of AMD include geographic atrophy in which there is patchy death of the retina and exudation in which abnormal neovascularization invades the subretinal or subretinal pigment epithelial space. Thus, preventing the accumulation of the inflammatory debris underneath the retina could be expected to alleviate much of the vision loss from this devastating disease.

1 Genes Associated with AMD

Two major pathways are well established in the pathogenesis of AMD. Both of these pathways were established using genetic methods. The first pathway to be established was the alternative pathway of complement. At present there is strong evidence for genetic variation in the genes encoding *complement factor H* (*CFH*) (Edwards et al. 2005; Hageman et al. 2005; Haines et al. 2005; Klein et al. 2005), variation at the *complement component 2/complement factor B* (*C2/CFB*) locus (Gold et al. 2006; Spencer et al. 2007), and the *complement component 3* (*C3*) locus (Maller et al. 2007; Yates et al. 2007). Recently variations in the gene encoding *factor I* (*CFI*) were reported but this has not yet been replicated or conclusively demonstrated to involve *CFI* beyond proximity (Fagerness et al. 2009). *Complement*

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factor H was the first gene in the alternative pathway to be associated with increased risk of AMD (Edwards et al. 2005; Haines et al. 2005; Klein et al. 2005). The Y402H variant in *CFH* tags a high risk haplotype associated with AMD in numerous studies. This variant is located in exon 9 and codes for an amino acid in the seventh short consensus repeat (SCR), a C-reactive protein (CRP) binding site. It is believed that the Y402H form of the protein causes alteration in binding of factor H to cell surface polyanions and CRP (Prosser et al. 2007; Ormsby et al. 2008). We demonstrated in our original 2005 paper (Edwards et al. 2005) that other *CFH* variants also show significant association with AMD and this has been confirmed by others (Li et al. 2006). It is unknown if these other SNPs are chance statistical associations that better capture the risk or if they tag variants of contributing to the biological risk.

The factor H protein is part of a regulatory network that suppresses alternative pathway activation. The alternative pathway is one of three enzyme cascades of the complement system. Constant activation of the alternative pathway occurs through spontaneous hydrolysis of C3 (Pangburn et al. 1981). Factor B binds this C3 hydrolysis product and surface bound C3b, and is cleaved into Ba and Bb by the serine protease factor D. This forms the C3 convertase (C3bBb) which initiates formation of the C5 convertase (C3bBbC3b), which in turn cleaves C5 and initiates formation of the membrane attack complex (SC5b-9) (Fig. 1). The anaphylatoxins C3a and C5a are formed during cleavage of C3 and C5. Complement factor H inhibits activation of the alternative pathway by accelerating the decay of the C3 convertase and along with factor I inactivates C3b by cleavage into iC3b and C3dg (Walport 2001).

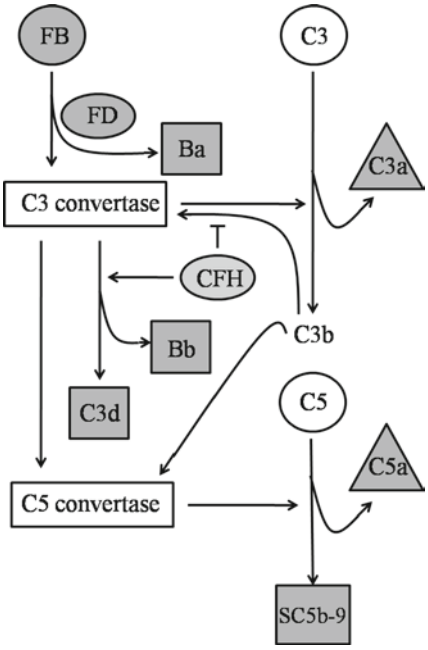

Fig. 1 Schematic depicting of the generation of anaphylatoxins C3a and C5a and SC5b-9 complex

The second major pathway associated with AMD is the age related maculopathy susceptibility 2 pathway (*ARMS2*) located on chromosome 10q26 (Jakobsdottir et al. 2005; Rivera et al. 2005). This region contains a hypothetical gene referred to as *LOC387715* and the *high temperature requirement A1* (*HTRA1*) gene. While the *HTRA1* gene is an excellent candidate for macular degeneration given its expression in the retinal pigment epithelium (Yang et al. 2006), there is at present no credible evidence that it is involved in AMD. The hypothetical gene *LOC387715* contains a truncating polymorphism at amino acid 38 (R38X), a substitution polymorphism (A69S), and an insertion-deletion in the 3′ untranslated region (Indel). These three variations are in very high linkage disequilibrium with the promoter polymorphism in *HTRA1*. Essentially there is a large haplotype block which contains all of these risk variants and many others that can be found in the online genetic variation databases. At this time genetic studies have not been able to distinguish between these multiple variations and identify the causal variation that leads to the increased risk for AMD. At present the best evidence for a functional change is that the presence of the insertion-deletion on this haplotype is thought to destabilize the RNA made from the two exons of the *LOC387715* gene (Fritsche et al. 2008). However, in vitro localization studies using over-expression have given inconsistent results between groups and as yet no group has convincingly demonstrated the presence of the native LOC387715 protein being produced by cells or in our bodies (Kanda et al. 2007; Fritsche et al. 2008; Kortvely et al. 2010; Wang et al. 2009). There is also concern that *LOC387715* may not be a coding gene because of the absence of a CpG site and other transcriptional features, and its presence only in recent primate evolution. It is our opinion that *ARMS2* should refer to the locus and not *LOC387715*, as appears to be the case in some online databases.

Many other genetic variants across the genome have been reported to be associated with AMD. Most of these probably represent type 1 or experimental errors and have not been replicated. However, a few deserve mention as possibilities. First, the *ApoE* gene has been implicated in AMD in several studies in which the *ApoE2* allele is the risk allele and the *ApoE4* that increases the risk of Alzheimers disease is protective (Souied et al. 1998; Schmidt et al. 2002; Baird et al. 2004; Zareparsi et al. 2004). The impact of this locus is relatively modest and unlike the alternative complement pathway and the 10q26 loci, the genetic variation in *ApoE* has not been observed in all subject groups. Promoter polymorphisms in the *vascular endotheial growth factor* (*VEGF*) gene have also been reported and there is some evidence for replication (Churchill et al. 2006; Haines et al. 2006). However, the evidence for this is also inconsistent across study groups. There have also been rare variations reported in the *ABCA4* (Allikmets et al. 1997; Allikmets 2000; Shroyer et al. 2001) and the *fibulin 5* gene (Stone et al. 2004) in association with macular degeneration. It is possible that mutations in these genes play a modest role in development of AMD. While this is important for gaining a pathophysiological perspective in understanding how drusen can develop, these variations are uncommon in the population and therefore may explain only a minute proportion of the overall disease burden. On the other hand, the opinion that large numbers of rare variants may contribute to common complex traits is becoming increasingly popular.

A number of large genome wide association studies are underway and there is hope that these studies will establish novel pathways for age related macular degeneration. Initial analyses of these datasets suggest a minimal role, if any, for common variation in these loci.

2 Non-Genetic Factors Increasing the Risk of AMD

In addition to genetic risks, lifestyle and demographic factors are important (Edwards and Malek 2007). Age plays a singularly critical role in the development of macular degeneration. It is possible for patients to develop AMD-like phenotypes in the setting of membranoproliferative gromerulonephritis type 2 or atypical hemolytic uremic syndrome, diseases which are also associated with variants in the *CFH* gene. However, in the absence of these types of rare diseases, age is a requirement for the development of macular degeneration. It is unknown whether some aspect of abnormal aging leads to susceptibility to the variation in the alternative pathway of complement and the *ARMS2* loci or whether the subtle changes in the established genetic pathways require decades to manifest themselves as disease. Smoking is also an important risk factor, particularly for development of exudative AMD (Despriet et al. 2006). Also there are a number of reports implicating the protective effects from the ingestion of micronutrients and increased risk from eating a high fat diet (Clemons et al. 2005). Indeed, animal models have been developed for AMD that utilize tobacco smoke or byproducts of tobacco smoke or high-fat diet (Espinosa-Heidmann et al. 2006; Fujihara et al. 2009). Thus, AMD is a classical complex trait in which a combination of genetic, demographic, and lifestyle choices contribute to the ultimate expression of disease and the development of the advanced complications of AMD (geographic atrophy, exudation) that are a leading cause of blindness in older individuals in developed countries (Klein et al. 1992).

3 Complement Proteins and AMD

In addition to the association of genetic variants in the alternative pathway of complement with AMD, there have been extensive immunohistochemical and proteomic studies demonstrating the deposition of complement proteins and their regulators in drusen (Johnson et al. 2001; Crabb et al. 2002; Zhou et al. 2006). These proteins include C3, C5, C5b-9, factor H and vitronectin among others. There is evidence that demographic and modifiable risk factors including age and smoking increase complement activation (Robbins et al. 1991; Pomeroy et al. 1997; Esparza-Gordillo et al. 2004). Further, a rare form of drusen associated macular degeneration called basal laminar drusen has been purported to have truncating mutations in the *complement factor H* gene (Boon et al. 2008). Thus, in addition to the genetic evidence there is extensive immunohistochemical and cell biological evidence for increased activation of complement in AMD.

Recently we and others have been able to explore the role of systemic activation of complement in AMD (Sivaprasad et al. 2007; Scholl et al. 2008; Hecker et al. 2010; Reynolds et al. 2009). The first report of increased complement activation in the blood of patients with AMD was by Sivaprasad et al. in 2007. In this small association study without replication they observed an increase in C3a protein levels in patients with AMD compared to controls. Given the requirement for replication in all association studies, their observation was of interest, but not conclusive. In a larger study by Scholl et al. in 2008 in which protein levels of several alternative pathway components were measured, an increase in substrate factor B, activating enzyme factor D, two split products (Ba, C3d) which are markers of chronic activation of the alternative pathway, the split products C3a and C5a which are markers of acute activation, and the downstream terminal complement complex (SC5b-9) was observed. The study by Scholl et al. was convincing because of the consistent observation of changes in multiple proteins, but also did not include an independent study group for replication. Because of these two studies, we sought to determine if these observations could be replicated and also to perform detailed analysis of genetic variants and haplotypes in the complement loci in order to understand how activation of the alternative pathway of complement is controlled in humans and in AMD.

We designed a functional genomic study in which we screened our database for patients with definite AMD and control subjects without AMD (Hecker et al. 2010). The definition of AMD is what we have used in our other studies and includes large drusen and a sufficient drusen area to fill a 700 μm circle and/or more advanced features (Edwards et al. 2005). We refer to these patients as drusen-associated AMD since some classification schemes include patients with pigmentary abnormalities but without drusen as AMD. Our control subjects were allowed to have five hard drusen but no more advanced features. A few hard drusen are present in almost all older subjects. The grading was based on both examination and color fundus photography for all subjects. We then screened our database for patients who had EDTA plasma samples available. Plasma used for these studies is collected under a stringent protocol in which the time from the blood draw to freezing of the processed plasma sample was required to be 6 hours or less. This is important, given that *in vitro* complement activation in plasma is known to occur over time at room temperature. The subjects were then screened for other diseases (e.g. rheumatoid arthritis) that might have excessive complement activation and these subjects were excluded. The resulting list of subjects included 125 subjects with AMD and 149 controls. There were no other criteria used to select these cases and controls.

In collaboration with Martin Oppermann's group, who has developed ELISA assays for alternative complement pathway proteins in humans (Oppermann et al. 1990; Oppermann et al. 1991b), we measured the plasma levels of complement factor B, factor D, the combination of complement factor H, a splice product of complement factor H (FHL-1) made up of the first seven SCRs (Estaller et al. 1991), and complement factor H related 1 (FHR-1), C5a, Ba, and C3d proteins. We also measured plasma creatinine levels because of the concern that renal function could be involved in the regulation of activation of complement (Oppermann et al. 1991a). We genotyped single nucleotide polymorphisms across the regulation of complement

activation (RCA) locus within which complement factor H is located, the C2/CFB locus, the C3 locus and the ARMS2 locus, corrected for covariates that alter plasma protein levels (age, gender, and plasma creatinine levels) and then performed a quantitative proteogenomic case-control association study. The goals of this study were first to determine if complement activation was under genetic control, and second to determine if the genetic risk contributing to the development of AMD also increased the activation of alternative pathway of complement in the blood.

Looking at uncorrected levels of plasma proteins in patients with AMD we observed that factor B, factor D, Ba and C3d were elevated in cases compared to controls. The most highly elevated protein was factor D (p-value = 0.0003). Thus, we were able to replicate the previous study by Scholl and colleagues and clearly demonstrate the elevation of substrates (factor B), activating enzymes (factor D), and split products indicative of increased activation of the alternative pathway of complement (Ba, C3d) in patients with AMD.

We observed that age, gender, and plasma creatinine levels impacted plasma levels of selected alternative pathway proteins. Because age is confounded with diagnosis and in order to minimize the loss of power from increasing the degrees of freedom, we elected not to include age, gender and creatinine as independent covariates in the analysis. Rather, we corrected the actual plasma levels for these three covariates by fitting a model to the controls using the three covariates as main effects. This allowed us to create a residual with the effect of age, gender, and creatinine levels removed. Next, in order to enable comparison of the effect of a change in plasma levels, we standardized the residual by subtracting the mean from it and divided the result by the standard deviation. Thus, we have created a type of score in which the odds ratio for a one standard deviation change in a particular protein can be readily compared to other proteins.

The odds ratio for an increase in factor B, D, Ba and C3d of one standard deviation ranged from 1.2 to 1.7. In the corrected model Ba was the most significantly associated with AMD and factor B was no longer statistically significant. Thus, after correction for covariates factor D, Ba, and C3d remain statistically significant. Taken together the results demonstrate increased systemic activation of complement in the blood of patients with AMD compared to controls.

We sought to understand how genetic variation increasing the risk of AMD was associated with the changes in plasma protein levels. Thus, we determined genotypes for variation across the *CFH*, *C3*, *C2/CFB* and *ARMS2* loci. The genotyping was sufficiently dense in order to capture the known variants associated with disease and also to capture the common haplotypes in each locus. Because the haplotypes represent the functional units of transcription and translation that regulate genes and code for proteins, our primary emphasis was on haplotype analyses.

However, Table 1 shows the effects of individual SNPs on plasma protein levels. It is important to note that these SNPs can occur in more than one haplotype and thus can represent combined effects of different protein forms or regulatory units. Particularly notable in the individual SNP observations was the valine 62 isoleucine polymorphism in exon 2 (rs800292) in the *CFH* gene in which there was a dramatic reduction in both Ba and C3d levels.

Table 1 The impact (*p*-values) of SNPs associated with AMD on complement protein levels in plasma. *P*-values are shown for corrected protein levels and represent the effect on plasma protein levels for one additional minor allele of a specific SNP and were obtained using quantile regression. The FH protein represents combined levels of factor H, factor H like 1 (FHL-1), and factor H related 1 (FHR-1) proteins

SNP	Gene	Function	FB	FD	FH	C5a	Ba	C3d
rs800292	*CFH*	Exon 2 (V62I)	0.2	0.7	0.3	0.3	7.0×10^{-6}	0.001
rs1061170	*CFH*	Exon 9 (Y402H)	0.9	0.5	0.7	1.0	0.1	0.5
rs1048663	*CFH*	Intron 9	0.9	0.7	0.8	0.9	0.8	0.7
rs2274700	*CFH*	Exon 10 (A473A)	0.9	0.7	0.2	0.7	0.05	0.2
rs412852	*CFH*	Intron 15	0.8	0.7	0.3	0.7	0.05	0.2
rs11582939	*CFH*	Intron 18	0.9	0.6	0.7	0.8	0.9	0.7
rs9332739	*C2*	Exon 7 (E318D)	0.07	0.1	0.4	0.6	2.0×10^{-6}	0.4
rs547154	*C2*	Intron 10	0.9	0.8	0.5	0.3	0.8	0.05
rs4151667	*CFB*	Exon 1 (L9H)	0.1	0.04	0.4	0.4	3.9×10^{-6}	0.4
rs2230199	*C3*	Exon 3 (R102G)	1.0	0.3	0.4	0.2	0.3	0.04
rs10490924	*ARMS2*	Exon 1 (A69S)	0.8	0.7	0.8	0.5	0.6	0.4

A similar reduction in Ba levels was seen for the *CFB* polymorphism leucine 9 histidine in the signal sequence of factor B (rs4151667). Of particular note in this result is that although the SNPs in *C2* and *CFB* are in extremely tight linkage disequilibrium (are inherited together as a block), the observation that polymorphisms in this region change factor Ba levels is consistent with the disease associated variation in AMD being secondary to the changes in factor B rather than C2. However, we have not yet had the opportunity to measure C2 levels in order to formally confirm this hypothesis. Also of note is the observation that the genetic variation in the *ARMS2* locus (10q26) was not associated with any changes in plasma complement levels suggesting that it is independent of complement pathway regulation.

Looking at the *CFH* locus, Fig. 2 shows the change in protein levels as a function of the four common haplotypes known as risk, protective 1, protective 2, and neutral. Interestingly, the risk haplotype encoding the factor H valine 62, 402 histidine allele had no difference in fluid phase activation of the alternative pathway of complement. This is not unexpected because the Y402H polymorphism is thought to regulate local tissue regulation of complement at the cell surface (Prosser et al. 2007).

The protective 1 haplotype coding the factor H 62 isoleucine and 402 tyrosine variants showed a dramatic reduction in both factor Ba and C3d levels. Claire Harris and colleagues have recently shown that the isoleucine variant of factor H binds C3 to a greater extent than the valine variant (Tortajada et al. 2009). Thus, the isoleucine protein variant encoded by the protective 1 haplotype is a better competitor for binding to C3 compared to factor B and would be expected to reduce the formation of the C3 convertase and therefore prevent the formation of factor Ba. This is indeed the effect we observed in the plasma of patients with and without AMD. It is important to note that we observed no differences in the extent of complement activation between cases and controls. The difference exists in the proportion of polymorphisms that lead to activation of complement in cases compared to controls. Thus, it appears that the genetic risks that lead to AMD are the same genetic variants that increase the activation of the

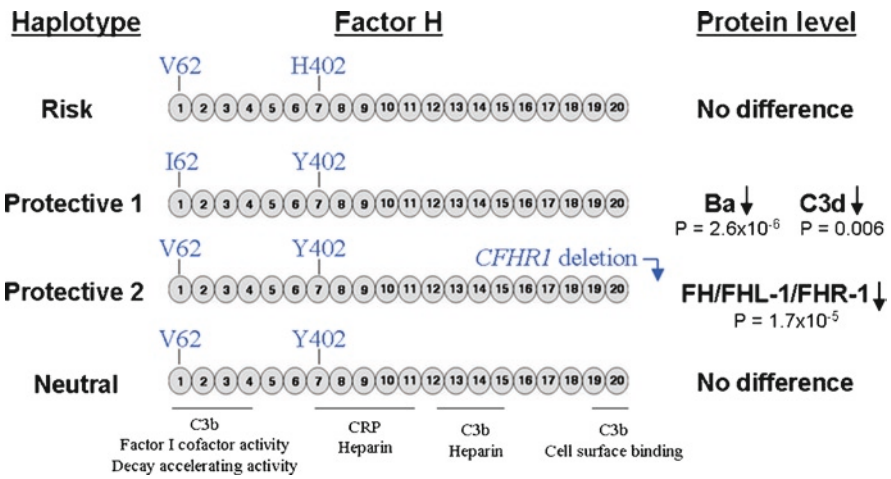

Fig. 2 Four common CFH haplotypes and their association with complement plasma protein levels. Haplotypes are identified by their commonly used designations of risk, protective 1, protective 2, and neutral. The 20 circles represent the short consensus repeats (SCRs) comprising the factor H protein. Each SCR is made up of approximately 60 amino acids connected by short linker regions. Locations of functional domains are indicated below the protein structures. Coding variants present in each haplotype are indicated in blue. The direction and significance of the haplotype associations with complement protein levels that we measured are indicated on the right-hand side of the figure

alternative pathway of complement. The antibody for complement factor H also recognizes the FHL-1 and FHR-1 proteins and serves as an internal control in our study. This is because the protective haplotype 2 contains a deletion of the *CFHR3* and *CFHR1* genes. In our study groups all subjects carrying the protective haplotype 2 carry this deletion. Thus, as expected, the combined FH/FHL-1/FHR-1 protein levels are decreased in subjects with the protective 2 haplotype. The neutral haplotype which has no impact on AMD risk showed no difference in plasma protein levels. This haplotype is essentially the same as the risk haplotype except for the absence of the histidine allele at position 402 thus encoding a factor H 62 valine, 402 tyrosine isoform. Thus, our observations of fluid phase complement activation are consistent with our understanding of the pathophysiology of the Y402H polymorphism and our observation on the protective haplotype 1 has been confirmed by *in vitro* biochemical studies.

Figure 3 shows the *C2/CFB* haplotypes and effects on protein levels in humans. We observed that the factor B haplotype coding for leucine at amino acid position 9 and arginine at position 32, which is the most common in humans, occurring in approximately 89% of subjects, increased C3d levels significantly. Indeed, in this locus the common haplotype is the AMD risk haplotype (Gold et al. 2006). The protective haplotype 1 encoding a factor B L9, Q32 form showed a marginal reduction in C3d levels which would require further study to determine if this is a valid observation. However, the protective 2 haplotype which contains the R32 polymorphism showed a highly significant reduction in Ba levels. Claire Harris has also shown that the R32 variants binds C3d with a fourfold higher affinity than the Q32 variant which can explain why the common risk haplotype increases complement activation (Montes et al. 2009). What is not clear is why the protective 2 haplotype coding factor B H9,

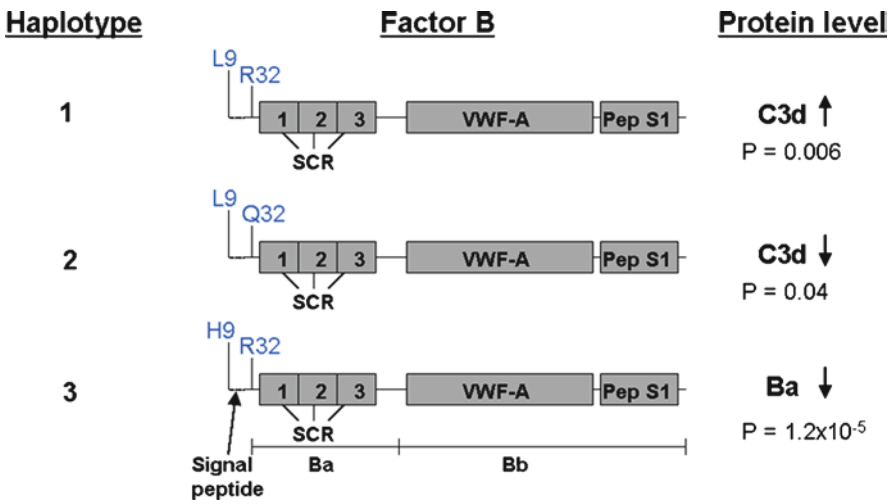

Fig. 3 Three most prevalent *C2*/*CFB* haplotypes and their association with complement plasma protein levels. Haplotypes are numbered in order of frequency. The three short consensus repeat regions (SCR) are indicated along with the von Willebrand factor, type A (VWF-A) and peptidase S1 (Pep S1) domains. The locations of the signal peptide region and the Ba and Bb fragments are also indicated. Coding variants present in each haplotype are shown in *blue*. The direction and significance of the haplotype associations with protein levels is indicated on the right-hand side of the figure

R32 is associated with decreased factor Ba levels. Further study is required in order to better understand the functional consequences of our observations.

We performed modeling studies to estimate the ability of the complement genetic variants and protein levels to predict AMD status. In stepwise regression models which did not include the *ARMS2* SNP we observed that the protein levels in the protein-only model accurately classified 67% of subjects, genetic variants in the genetic model correctly classified 70% of subjects, while the combined protein-genetic model correctly classified 74% of subjects. When the *ARMS2* SNP was added to the model the accuracy of the combined model increased to 79%, consistent with previous modeling studies. Thus, our data suggest that the genetic risks explain much of the changes in plasma protein levels and remain a better predictor of AMD risk than the plasma protein models. This is opposite of the conclusion that Scholl and colleagues obtained (Scholl et al. 2008), and we believe this is because of the inclusion of age in the model as an independent covariate which can be confounded with diagnosis and also impacts plasma complement levels. We believe that by removing the effect of age and the other covariates prior to the modeling studies, the impact of these covariates can be more accurately managed.

We and others have shown that systemic complement activation as indicated by factor Ba and C3d increases the risk of having AMD in case-control studies (Scholl et al. 2008; Hecker et al. 2010). Factor D is also increased by an as yet unknown mechanism as there is no evidence that polymorphisms in this locus are associated with AMD. It is particularly notable that different haplotypes in the *CFH* and *CFB* regions had distinct mechanisms of altering these risks. The *CFH* risk, neutral, and protective 2 (P2) haplotypes probably have no affect on plasma

activation. This suggests a local action for the risk haplotype. The neutral haplotype may have no impact on complement levels relative to the other haplotypes. In our studies we have not observed an independent effect of the P2 haplotype unlike the initial study reported by Anne Hughes (Hughes et al. 2006). The effect of the P2 haplotype can be explained in our subjects by the absence of the risk haplotype alone. On the other hand, the P1 haplotype showed a dramatic reduction in complement activation as indicated by factor Ba levels. This has been nicely corroborated with functional studies as described above. Finally, our modeling studies suggest that genetic risks explain much but not all of the excess complement activation in patients with AMD compared to controls.

4 Conclusions

Our study has important implications for quantitative functional genomic studies in general and for the pathogenesis of AMD. First, we observed that while statistical modeling was useful for creating a risk model of the minimum number of variants, it did not detect the independent effect that we observed on plasma complement levels. Thus, we would caution investigators not to over interpret the statistical models. That is, while the statistical modeling is very useful for creating the minimal number of genetic variants that predict disease risk, it may fail to elucidate, or even discard, the underlying functional variants that are important in understanding the pathophysiology of a complex trait.

Second, we have shown that the activation of the alternative pathway of complement is under genetic control in humans. We observe that this same genetic control equates with the genetic risk that increases the risk for AMD. This strongly argues that there is an important role for ongoing, life-long, increased activation of complement in tissue fluids in the pathogenesis of AMD. Our observations also suggest that some of the risks in the alternative pathway of complement are not needed for the fluid phase, suggesting a dual model in which ongoing over-activation in tissues and local tissue dysregulation of complement activation play an important role in the development of AMD.

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Chapter 5
Bisretinoids of RPE Lipofuscin: Trigger for Complement Activation in Age-Related Macular Degeneration

Janet R. Sparrow

Abstract Genetic association studies and investigations of the constituents of subretinal deposits (drusen) have implicated complement dysregulation as one factor predisposing individuals to increased risk of age-related macular degeneration (AMD). Here we review evidence that molecular fragments released by photooxidation of the bisretinoids of retinal pigment epithelial lipofuscin, can activate complement. Complement activation by this mechanism is dependent on the alternative pathway. The diretinal conjugates comprising RPE lipofuscin accumulate in the cells throughout the lifetime of an individual. As such, these photooxidative processes, in a setting of complement dysregulation could contribute to chronic inflammation underlying AMD pathogenesis.

1 RPE Lipofuscin and Macular Degeneration

Throughout the lifetime of an individual, retinal pigment epithelial (RPE) cells in the eye accumulate autofluorescent bisretinoid-adducts that are stored within lysosomal organelles and that constitute the lipofuscin of the cell. These pigments originate in photoreceptor cells and result from reactions of all-*trans*-retinal, the product of 11-*cis*-retinal photoisomerization (Sparrow and Boulton 2005). Individuals vary in terms of the extent to which these bisretinoid are accumulated but the most pronounced accretion occurs in the RPE cells of central retina (Delori et al. 2001). The deposition of this material is responsible for fundus autofluorescence, the natural autofluorescence of retina that can be imaged by scanning laser ophthalmoscopy (Delori et al. 2007). Indeed the extensive systems of conjugated double bonds exhibited by these retinoid-derived fluorophores enable excitation and fluorescence emission at wavelengths within the visible region of the spectrum (Sparrow 2007). A2E and its isomers were the first of the bisretinoids of RPE lipofuscin to be characterized

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(Eldred and Lasky 1993; Sakai et al. 1996; Parish et al. 1998; Ben-Shabat et al. 2002b); others we have isolated are the all-*trans*-retinal dimer series and A2-DHP-PE (Fishkin et al. 2005; Kim et al. 2007).

Not surprisingly given their origin from retinoid, these fluorescent pigments are both photosensitizers for and quenchers of reactive forms of oxygen, the result being that the pigments undergo photooxidation at carbon-carbon double bonds (Sparrow et al. 2000, 2002; Ben-Shabat et al. 2002a; Dillon et al. 2004; Kim et al. 2008) (Fig. 1). The oxygen-containing moieties that form along the side-arms of these bisretinoids include three membered rings that include one oxygen atom (epoxide; C–O–C); heterocyclic rings of four carbons and one oxygen (furan) and heterocycles that incorporate four carbons and an endoperoxide (O–O) into the ring. Photo-induced cleavage of the bisretinoids at these oxygen-containing groups also occurs (Fig. 1). All of these lipofuscin pigments and some of their oxidized forms have been detected and measured in human RPE and in eyecups of mice having null mutations

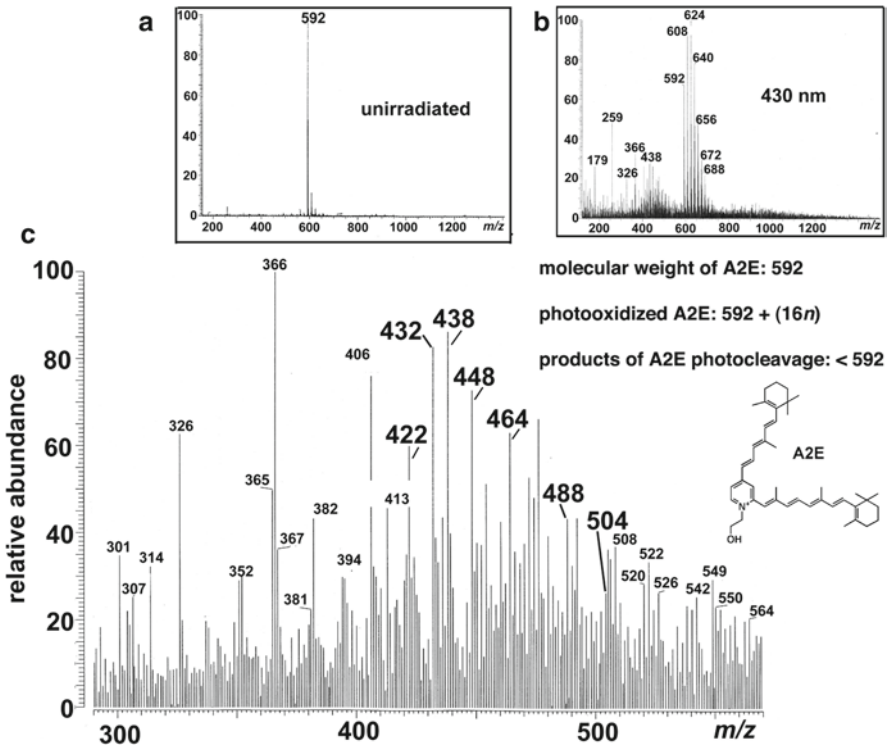

Fig. 1 ESI mass spectrometry analysis of 430 nm exposed A2E. Samples of A2E (*m/z* 592) were unirradiated (**a**) and irradiated at 430 nm (**b**); (**c**) expanded view of panel (**b**) in the range of *m/z* 300–540. Peaks differing by *m/z* +16 (*m/z* 608, 624, 640, 656, 672, 688) reflect photooxidation at carbon-carbon double bonds (**b**). Lower mass peaks (<*m/z* 592) correspond to A2E fragments produced by photooxidation-induced cleavage (**b**, **c**); m/z values for some of the more prominent photo-cleavage products are presented in *bold text*

in *Abca4/Abcr* (Parish et al. 1998; Kim et al. 2004, 2007; Jang et al. 2005a), the gene responsible for recessive Stargardt disease (Allikmets et al. 1997).

2 Age-Related Macular Degeneration and the Complement System

There has often been speculation of a link between RPE lipofucin and the extracellular deposits that form subretinally as drusen; drusen serve as a risk factor for age-related macular degeneration (AMD) (Davis et al. 2005). Studies of the composition of drusen have also revealed the presence of several complement related proteins and inflammatory factors such as C-reactive protein (Hageman et al. 2001; Anderson et al. 2002; Crabb et al. 2002; Donoso et al. 2006; Nozaki et al. 2006). Consistent with these findings, genetic association studies have demonstrated links between DNA sequence variants in complement factor H (CFH), factor B (BF)/complement component C2 and complement component C3, and protection or risk for AMD (Conley et al. 2005; Edwards et al. 2005; Hageman et al. 2005; Haines et al. 2005; Klein et al. 2005; Zareparsi et al. 2005; Gold et al. 2006; Yates et al. 2007). Together these proteomic and genetic studies implicate complement dysregulation in the presence of an activating agent, as an underlying cause of AMD pathogenesis in a significant number of cases.

3 Complement Activation by Photoproducts of the RPE Bisretinoid A2E

Prompted by an interest in understanding stimuli responsible for complement activation leading to AMD, we considered the possibility that products of the photooxidation of RPE bisretinoids serve as activators of the complement system – activators that would predispose the macula to disease, and that could generate the low grade complement activation that overtime contributes to chronic inflammatory processes. Not only do the bisretinoid compounds have unique structures, but the photooxidation has the potential to generate a complex mixture of cleavage fragments that could be recognized by the complement system as foreign. Moreover, findings in our cell culture models suggest that while RPE lipofuscin pigments are otherwise sequestered within the lysosomal compartment of the cell, photooxo-A2E can redistribute inside and outside the cell, secondary to fragmentation (Zhou et al. 2005).

Accordingly, we constructed in vitro assays employing normal human serum as a source of complement. As a first approach we used a cell-based assay that provides for a population of RPE cells that had accumulated A2E in culture and a population of RPE cells without A2E (Sparrow et al. 1999). The A2E molecule consists of two retinoid-derived arms connected through a pyridinium ring (Fig. 1) and exhibits absorbance maxima at ~330 and 440 nm. When A2E-containing ARPE-19 cells that had accumulated A2E in culture were irradiated (430 nm) to generate photooxidized

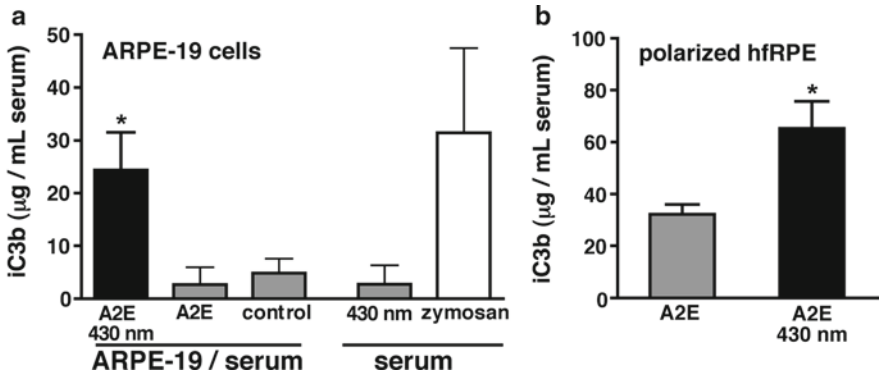

Fig. 2 The C3 cleavage product iC3b is elevated in serum placed in contact (37°C for 2 h) with RPE that had accumulated A2E and are irradiated at 430 nm to generate A2E photooxidation products. (**a**) ARPE-19 cells; (**b**) polarized fetal human RPE (fhRPE); serum placed on basal side of cells. Enzyme immunoassay. Zymosan incubated in serum at 37°C served as positive control. Values from undiluted human serum incubated in empty wells at 37°C were subtracted as background. Means ± SEM, 3–8 experiments; $*p < 0.05$ as compared to controls (*grey bars*)

fragments of the di-retinoid we found that levels of iC3b and C3a-desArg, fluid-phase products of C3 activation, were elevated relative to controls (Zhou et al. 2006b) (Fig. 2a). The levels of iC3b were similarly elevated on the basal side of polarized cultures of human fetal RPE that had internalized A2E and were irradiated (Fig. 2b). We considered the possibility that irradiation-induced cell apoptosis could serve as a trigger for complement activation in these assays rather than A2E photooxidation. Nevertheless, we found that apoptosis induced with etopiside, an inhibitor of the enzyme topoisomerase II, did not lead to increased iC3b generation in serum overlying the cells. This result indicated that apoptosis per se does not activate complement (Zhou et al. 2009).

To test for complement activation under conditions involving direct contact between complement proteins in serum and oxidized forms of A2E, we synthesized peroxy-A2E and furano-A2E (Jang et al. 2005a) for introduction to serum. By enzyme immunoassay, iC3b was measured in abundance when normal human serum was incubated in wells precoated with peroxyA2E and furanoA2E as compared to vehicle control (Fig. 3a). Conversely, no difference in iC3b content was observed when serum was incubated in wells coated with A2E.

4 Complement Activation by Oxidized All-*trans*-Retinal-Dimer

A2E is not the only bisretinoid compound of RPE lipofuscin. For instance, we have identified a family of pigments that includes a condensation product of two all-*trans*-retinal, all-*trans*-retinal dimer, and two conjugated forms of all-*trans*-retinal

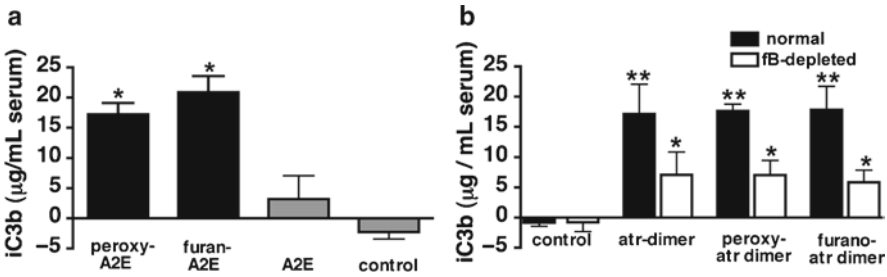

Fig. 3 iC3b generated in serum incubated in contact with oxidized forms of A2E (peroxy-A2E and furano-A2E) and all-*trans*-retinal dimer (atr dimer). (**a**) Normal human serum was incubated in wells of a microtiter plate pre-coated with peroxy-A2E, furano-A2E. Means ± SEM of three experiments. *$p < 0.05$ versus A2E and control (sham coating). (**b**) iC3b generation in serum incubated with atr dimer and two oxidized forms, peroxy-atr dimer and furano-atr dimer is dependent on presence of factor B. Normal serum was substituted with serum depleted of factor B. **$p < 0.05$ versus control (sham coating) *$p < 0.05$ versus normal serum. iC3b was measured by enzyme immunoassay; levels in serum incubated in an empty well at 37°C were subtracted as background

dimer (all-*trans*-retinal dimer-ethanolamine and all-*trans*-retinal dimer-hosphatidylethanolamine). Significantly, unconjugated all-*trans*-retinal dimer is a more potent generator of singlet oxygen than A2E and also quenches singlet oxygen more efficiently (Jang et al. 2005a; Kim et al. 2007). Accordingly, photooxidized forms of all-*trans*-retinal dimer are particularly prominent in RPE lipofuscin (Kim et al. 2007). Thus we also undertook to test these photoproducts as activators of complement. Peroxy-all-*trans*-retinal dimer and furano-all-*trans*-retinal dimer were synthesized as previously described (Kim et al. 2007) and were applied to microtiter wells. Incubating the coated wells with serum as a source of complement advanced the generation of iC3b (Fig. 3b).

5 Complement Activation by Bisretinoid Photoproducts is Dependent on Factor B

Within the alternative complement pathway, an amplification loop is established when C3 is cleaved to C3a and C3b, and factor B, after binding to C3b is cleaved to Bb (and Ba). Bb contains a serine protease domain and remains bound to C3b thereby forming the C3 convertase C3bBb. Two variants in CFB, R32Q and L9H are reported to confer reduced susceptibility to AMD, although due to strong linkage disequilibrium in the CFB/C2 region on chromosome 6 it has been difficult to assign the disease association to the CFB versus C2 gene (Spencer et al. 2007). Interestingly, however, in vitro functional assays have shown that the FB 32Q variant exhibits considerably reduced affinity for C3b than does the 32Q allele. This finding suggests that the protection against AMD may be associated, at least in part, with the 32Q variant in fB and the mechanism may involve reduced propensity for formation of the amplifying C3 cleavage enzyme (Montes et al. 2009). Given these findings, we were

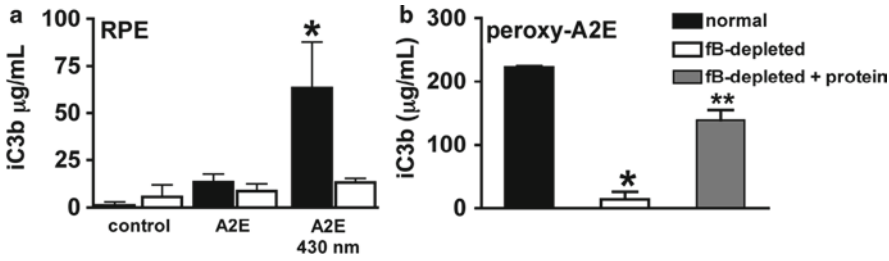

Fig. 4 Production of the C3 cleavage product, iC3b, in response to A2E photooxidation is dependent on presence of complement component factor B. (**a**) iC3b levels in normal human serum or factor B-depleted serum overlying RPE cells that had/had not accumulated A2E and were irradiated/not irradiated to generate A2E photooxidation products; control, cells only. iC3b was measured by enzyme immunoassay; levels in serum incubated in an empty well at 37°C were subtracted as background. *$p<0.01$. (**b**) Depletion of factor B attenuates iC3b production in serum incubated in wells precoated with peroxy-A2E; inhibition is reversed when factor B protein is added to the depleted serum. Mean ± SEM of 2–4 experiments, duplicate wells per experiment. *$p<0.01$ as compared to normal serum; **$p<0.05$ as compared to factor B-depleted serum

interested to test FB in our system. Using our assays we replaced normal human serum with FB-depleted serum and found that the increase in iC3b observed in association with irradiation of A2E-laden ARPE-19 cells was abrogated (Fig. 4a). Elevations in iC3b generated by zymosan, a component of yeast cell walls that was used as a positive control in these experiments, were also prevented in the absence of factor B. Similarly, when exposed to FB-depleted serum, oxidized forms of A2E, in particular peroxy-A2E and furano-A2E also failed to generate elevated levels of iC3b; with normal human serum under the same conditions, iC3b was increased (Fig. 4b). The inhibition observed in the presence of FB-depleted serum was reversed by adding back factor B protein (Fig. 4b). These findings are readily explained by failure of the alternative pathway feedback loop in the absence of FB.

6 C-Reactive Protein Modulates Complement Activation by RPE Bisretinoids

Risk of AMD is also associated with elevated serum levels of C-reactive protein (CRP) (Seddon et al. 2004; Boekhoorn et al. 2007; Schaumberg et al. 2007), a plasma protein that is considered to have both pro- and anti-inflammatory properties. CRP levels in serum are generally less than 3 µg/mL (Black et al. 2004). In an acute infection, CRP levels in serum can increase by as much as a 1,000-fold and CRP can promote concentration dependent complement activation by forming complexes involving both ligand and C1q of the classical pathway (Laine et al. 2007). Under conditions of chronic low grade inflammation, minor elevations in CRP are also observed (3–10 µg/mL) (Black et al. 2004); for instance, in AMD 90% of serum CRP values have been reported to be at or below 10 µg/mL (Seddon et al. 2004).

The role of CRP here is less clear. Our system allowed for testing the effects of CRP at moderate levels. Thus we added exogenous CRP to normal human serum at concentrations of 1 and 10 μg/mL and found that as monitored by iC3b levels, complement activation was suppressed when serum was incubated with oxidized forms of A2E. This negative regulation would be consistent with CRP binding to the SCR 7 domain of CFH such that the alternative pathway amplification loop was suppressed. Indeed, the Y402H variant sits in the SCR 7 domain of CFH and experiments indicate that the AMD-associated 402H variant confers reduced binding of CFH to CRP, a lowering of affinity that is expected to impair CRP-mediated negative regulation of the alternative pathway and could thus be permissive for chronic inflammation (Laine et al. 2007; Sjoberg et al. 2007; Ormsby et al. 2008). Since the serum donor in our experiments expressed the CFH-402Y variant, CFH-CRP interaction would have been functional.

7 Suppression by POT-4, a C3 Cleavage Inhibitor

Compstatin is a 13-amino acid cyclic peptide that inhibits C3 activation (Sahu et al. 1996). Suppression of C3 cleavage by compstatin is more potent in the alternative pathway probably because the compound also binds to C3b. Optimization of compstatin activity has lead to generation of the analog POT-4 (Ricklin and Lambris 2007), a compound that is currently the subject of clinical trials aimed at investigating safety and efficacy when delivered via intravitreal deposition in patients with atrophic AMD. As expected, POT-4 was an efficient inhibitor of A2E photoproduct-induced complement activation. In terms of therapeutics, inhibition of C3 is widely applicable, but the suppression of adverse complement activity without compromising the defensive functioning of the complement system, will be a challenge (Ricklin and Lambris 2007).

8 Summary

The disease processes leading to AMD likely evolve gradually over several years. Complement activation by photoproducts released from RPE lipofuscin could constitute a relatively early event that in the setting of complement dysregulation would continue unabated over several years. Invading pathogens have been suggested as initiating agents in complement-mediated events underlying AMD (Hageman et al. 2005). Some case-control studies have found an association with exposure to *C. pneumoniae* (Ishida et al. 2003; Kalayoglu et al. 2003). It has also been reported that subjects homozygous for the risk allele of CFH (CC) and who also presented with the upper tertile of antibody titres to *C. pneumoniae* had the highest increased odds of disease progression (11.8-fold) as compared to those with the lowest tertile of antibody titer and the TT genotype (Baird et al. 2008). Nevertheless, other studies

have not demonstrated an association (Robman et al. 2005; Haas et al. 2009). It is also not clear that infection can readily explain why the disease process has a predilection for the macula.

The finding that a number of naturally occurring antioxidants, including vitamins E and C, anthocyanins and sulforaphane offer protection against A2E photooxidation (Sparrow et al. 2003; Jang et al. 2005b; Zhou et al. 2006a) is consistent with the notion that oxidative mechanisms contribute to disease pathogenesis in AMD. For instance the Age-Related Eye Disease Study (AREDS) reported that high doses of the antioxidants vitamin E and C and zinc reduce the progression from intermediate to advanced AMD (AREDS 2001). Other studies of antioxidant intake report similar findings (Mares-Perlman et al. 1996; Christen et al. 1999; Delcourt et al. 1999; AREDS 2001; van Leeuwen et al. 2005). Other risk factors, such as smoking (Smith et al. 1996; AREDS 2000) and light exposure (Taylor et al. 1992; Cruickshanks et al. 2001; Tomany et al. 2004) may operate via and/or exacerbate oxidative processes. Interestingly, Wu et al., observed that exposing RPE to the oxidant hydrogen peroxide, attenuated IFN-g-induced stimulation of CFH (Wu et al. 2007), an important modulator of complement activation.

In summary, we propose that products generated by the photooxidation of RPE lipofuscin pigments serve as activators of the complement system – activators that would predispose the macula to disease, and that could generate the low grade complement activation that overtime contributes to the chronic inflammatory processes.

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Chapter 6
The Role of the Classical Complement Cascade in Synapse Loss During Development and Glaucoma

Allison M. Rosen and Beth Stevens

Abstract Glaucoma is one of the leading causes of vision loss worldwide, yet the signals that initiate the progressive degeneration of optic nerve axons and the selective loss of retinal ganglion neurons (RGCs) remain elusive. Reactive gliosis, release of inflammatory cytokines, and complement upregulation all occur in the early stages of glaucoma in several disease models. Recent work has implicated the classical complement cascade in the elimination of excess synaptic connections in the developing visual system and in early synapse loss associated with glaucoma, suggesting that mechanisms of developmental synapse elimination may be aberrantly re-activated in glaucoma. This review describes current evidence in support of this "synaptic" hypothesis and places complement in the context of other well-described mechanisms of neurodegeneration occurring in the glaucomatous eye.

1 Introduction

The elimination of excess synaptic connections during postnatal development is a critical process in the formation of functional neural circuitry. While synaptic pruning is necessary for proper synaptic wiring in the immature brain, synapse loss and dysfunction have emerged as a hallmarks of several neurodegenerative diseases in the mature nervous system (Selkoe 2002). Glaucoma is one of the leading causes of blindness in the aged population, yet the signals that trigger neurodegeneration and the selective loss of retinal ganglion cell neurons (RGCs) remain elusive. Recent research indicates that a common feature in neurodegenerative diseases is the loss of functional, mature synapses early in the pathological progression of the disease (Selkoe 2002; Stevens et al. 2007). Thus, investigating the mechanism by which synapses are eliminated is important in advancing the understanding of retinal degeneration and offers potential targets for early therapeutic intervention.

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Recent work has implicated glia and the classical complement cascade in developmental synapse elimination and in synapse loss associated with glaucoma, suggesting that a common mechanism is regulating both processes (Stevens et al. 2007). The primary role of the complement cascade in the innate immune system is to opsonize or "tag" unwanted cells or debris for rapid elimination. C1q, the initiating protein of the classical cascade, is significantly upregulated in developing neurons in response to a glial-derived signal. Moreover, C1q and downstream C3 are localized to developing synapses, and mice deficient in these molecules exhibit significant and sustained defects in CNS synapse elimination in the developing visual system (Stevens et al. 2007). Interestingly, several of the key events in developmental synapse elimination reappear in glaucoma. Reactive gliosis, release of inflammatory cytokines, and complement upregulation all occur in the early stages of glaucoma in several disease models. In a mouse model of glaucoma, C1q becomes upregulated and synaptically localized in the retina very early in the disease (Stevens et al. 2007). Together these observations suggest a model by which complement cascade proteins "tag" CNS synapses for elimination in the developing and diseased nervous system. This review discusses recent evidence demonstrating how glial cells, and the classical complement cascade may be activated in glaucoma and fit into the well-described mechanisms of neurodegeneration occurring in the glaucomatous eye.

2 Current Opinion on Glaucoma

Glaucomas are a group of neurodegenerative diseases that affect the eye and result in blindness caused by progressive degeneration and loss of retinal ganglion cells (RGCs). Onset of the disease is often associated with elevated intraocular pressure (IOP), yet the development of glaucoma in the absence of high IOP, termed normal tension glaucoma (NTG), is observed with an incidence rate similar to high IOP glaucoma (Johnson and Tomarev 2009). While there are several theories as to how high IOP could trigger the events that lead to RGC death and blindness, these cases of NTG suggest that IOP is not the only stressor promoting the onset of glaucoma. Also, while high IOP is often associated with glaucoma, elevated IOP does not necessarily result in RGC degeneration. Since IOP elevation is not always a reliable predictor for glaucoma, recent studies have been geared toward answering two big questions: What are the earliest indicators of glaucoma? What are the molecular mechanisms underlying disease progression and pathology? Current evidence suggests complement and neuro-immune interactions may be central to the development and progression of the disease.

3 Animal Models of Glaucoma

Some clues to the early initiating events of glaucoma have come from animal models of the disease. There are diverse rodent models for glaucoma that fall into two main categories: (1) Genetic models, (2) Experimentally-induced models. Although each of these models has its caveats, together they have provided important insight

Table 1 Rodent models of glaucoma

Glaucoma type	Models	Genetic/ experimental	Key references
Ocular hypertensive	DBA/2J	Genetic	Libby et al. (2005a)
	Myocilin mutant	Genetic	Alward et al. (1998)
	Collagen type 1 mutant	Genetic	Mabuchi et al. (2004)
	Laser-induced ocular hypertension	Experimental	Grozdanic et al. (2003)
	Episcleral vein cauterization	Experimental	Ruiz-Ederra and Verkman (2006)
	Hypotonic saline injection	Experimental	Morrison et al. (1997)
Ocular normotensive	Ischemia/reperfusion	Experimental	Büchi et al. (1991)
	GLAST knockout	Genetic	Harada et al. (2007)
	EAAC1 knockout	Genetic	Harada et al. (2007)
	Endothelin-1 infusion	Experimental	Chauhan et al. (2004)
	Optic nerve crush	Experimental	Levkovitch-Verbin et al. (2000)
	Excitotoxic agent injection	Experimental	Vorwerk et al. (1996)
Developmental	Cyp1b1	Genetic	Libby et al. (2003)
	Foxc1/c2	Genetic	Smith et al. (2000)
	Bmp4	Genetic	Chang et al. (2001)
	Pitx2	Genetic	Phillips (2000)

into the onset and progression of glaucoma. As glaucomas represent a group of neurodegenerative diseases with a spectrum of symptoms and stages, several parallel approaches using multiple models are necessary to elucidate different aspects of the disease.

Genetic strains have been particularly informative since many of these strains show a gradual onset of glaucoma symptoms (Table 1). Several genetic strains, including the DBA/2J mouse, myocilin mutants, and collagen type-1 mutants, feature elevated IOP (Johnson and Tomarev 2009). The DBA/2J mouse has been studied extensively. This strain has recessive mutations in two genes: *Gpnmb* (glycosylated protein nmb) and *Tyrp1* (tyrosinase related protein 1) (Libby et al. 2005a; Howell et al. 2007b, 2008). These mutations cause an abnormality in the melanosomes of the iris resulting in an inflammatory response directed against the iris (Libby et al. 2005b). In addition, this model exhibits progressive retinal ganglion cell degeneration leading to eventual RGC loss, and most of the hallmark pathologies seen in human glaucoma (Buckingham et al. 2008). The genetic background of this strain predisposes these animals to elevated IOP, death of retinal ganglion cells, and atrophic excavation of the optic nerve (Libby et al. 2005a). Other genetic mouse models of glaucoma, such as mice deficient in the glutamate transporters, GLAST and EAAC1, recapitulate symptoms of glaucoma not associated with high IOP (Harada et al. 2007). Both GLAST and EEAC1 glutamate transporters as well as glutamate receptors are downregulated in glaucomatous eyes (Naskar et al. 2000). These strains are useful tools for investigating alternative mechanisms of RGC death not triggered by IOP elevation. For example, studies using EAAC1-deficient mice

revealed that RGCs are more sensitive to oxidative stress in the absence of glutamate transporters (Harada et al. 2007). In addition, reduced expression of these transporters can cause a buildup of glutamate in the eye. Astrocytes are the main cells responsible for clearing glutamate from the extracellular space via glutamate transporters. When astrocytes become activated in glaucoma, they downregulate glutamate transporter expression, suggesting a mechanism by which astrocyte or glial activation can promote RGC death via excitotoxicity (Naskar et al. 2000). Consistent with this finding, treatment with glutamate receptor antagonists prevents RGC loss in glaucoma models (Chaudhary et al. 1998).

Experimentally induced models for glaucoma have been developed by artificially elevating intraocular pressure, crushing the optic nerve, or treating the eye with excitotoxic agents. In rats and non-human primates, IOP elevation can be experimentally induced using a laser to cauterize the veins draining the eye or by other approaches (Table 1). (Johnson and Tomarev 2009). In addition to IOP-dependent experimental models, optic nerve crush experiments model the hypothesis that glaucoma may be caused by an initial insult to RGC axons. Alternatively, adding excitotoxic agents to the eye can be used to analyze the later stages of glaucoma when RGCs degenerate and die presumably due to glutamate build up (Berkelaar et al. 1994; Dreyer et al. 1996). These techniques also have proven useful for identifying neuroprotective versus neurotoxic factors (Schwartz 2004), and for following the progression of glaucoma after high IOP and/or axonal damage is first induced. They can be used to study the preclinical and early phases of glaucoma in a more controlled way than can be done with genetic strains that develop the disease with a gradual, variable progression. Experimentally-induced models, however, generally do not exhibit all symptoms of glaucoma and cannot elucidate possible mechanisms behind IOP elevation or the very earliest events in glaucoma.

4 Pathological Progression of Glaucoma

Using various animal models as well as in vitro approaches, several critical factors necessary for RGC survival as well as signals that promote RGC degeneration and death have been identified. Recent work has focused on understanding the early stages of the disease and connecting the preclinical hallmarks of glaucoma to the onset of RGC degeneration. A combination of genetic and experimentally induced animal models of glaucoma has been integral to the study of the progression of glaucoma from very early preclinical stages through the late phases of RGC death. Here we summarize the known cellular and molecular hallmarks of each stage of glaucoma with particular emphasis on the role of complement and neuro-immune interactions based on research from various animal and experimental models (Table 1) as well as clinical evidence from human patients. Each of these hallmarks is discussed in more detail later in this review.

Preclinical. Risk factors that can predispose humans to developing glaucoma include diabetes, hypertension, cardiovascular diseases, and genetic factors including

mutations in the genes *myocilin* and *optineurin* (Rezaie et al. 2002; Fan et al. 2004). One of the most prominent risk factors for developing glaucoma in humans is elevated intraocular pressure, though the mechanisms by which IOP initially becomes elevated are not clearly understood. Further, treatments to lower IOP are not always successful in eliminating glaucomatous symptoms. Only one-third to one-half of all glaucoma patients presents with high IOP levels (Renu et al. 2009). Another 30–40% of glaucoma patients do not have elevated IOP and are diagnosed with normal tension glaucoma (NTG) (Renu et al. 2009). These results suggest that mechanisms independent of IOP elevation contribute to the onset of glaucoma. Some of these IOP-independent risk factors have been identified. For example, recent findings that endothelin-1, a vasoconstrictor, is elevated in the aqueous humor and plasma of glaucoma patients suggests that vascular abnormalities underlie NTG cases (Chauhan et al. 2004). Another alternative early indicator of RGC abnormality prior to the detection of visual loss is an alteration in higher visual areas such as LGN and visual cortex (Yücel et al. 2003; Duncan et al. 2007). PET scans revealed abnormal patterns of activity in these regions after experimentally inducing glaucoma in primates (Imamura et al. 2009). These alterations could be detected prior to any detectable alterations in the optic disc by fundoscopic examination, suggesting that an alteration in RGC connectivity or synapse loss may be one of the earliest events in glaucoma.

Early glaucoma. In humans, the early stages of glaucoma are marked by limited loss in the peripheral visual field. In experimental and genetic models of the disease in which progression of the disease can be monitored more thoroughly, changes astrocyte proliferation/activation, upregulation and activation of components of the complement cascade (C1q, C4, C3) (Stasi et al. 2006; Steele et al. 2006; Stevens et al. 2007), and synapse loss have been observed prior to substantial defects in visual acuity in several models (Duncan et al. 2007; Fu et al. 2009). Astrocyte activation is one of the earliest events occurring in response to elevated IOP in experimental models (Johnson et al. 2000). Recent work investigating the early stages of glaucoma in the DBA/2J mouse has also provided evidence that an insult to RGC axons exiting the eye through the lamina cribrosa, which is ensheathed by astrocytes, is one of the earliest events in glaucoma (Howell et al. 2007a; Daniel et al. 2009). Elevated IOP could lead to this axonal damage as could activation of astrocytes in the lamina.

Mid-stage glaucoma. As glaucoma progresses, RGC death begins in the periphery and spreads toward the center of the visual field. Reactive astrocytes are still present in the glaucomatous eye at this stage and microglia proliferation and activation become evident. Microglia are thought to underlie the patchy loss of RGCs seen at this phase of the disease (Neufeld 1999). Concurrent with the proliferation and activation of microglia, the production of inflammatory cytokines such as TNF-α and IL-1β are increased at this stage (Yuan et al. 2000; Zhong et al. 2007). These inflammatory cytokines could disrupt the blood brain barrier and allow additional immune cells to infiltrate the eye (Tezel et al. 2008). Expression of complement genes (evidence for C1q, C4, and C3 described below) continues to increase and these proteins localize to the inner plexiform layer (IPL) in the DBA/2J mouse (Stevens et al. 2007).

Late glaucoma. Late stage glaucoma is characterized by widespread RGC death and eventual blindness. Widespread glial proliferation, and particularly microglial activation and proliferation, is thought to contribute to this process through sustained release of inflammatory cytokines such as TNF-α that promote apoptosis (Nakazawa et al. 2006). In addition, downregulation of glutamate transporters in reactive astrocytes is hypothesized to promote RGC death via excitotoxicity at this late stage. RGCs are known to die via apoptosis (Dreyer et al. 1996; Naskar et al. 2000), and in the DBA/2J mouse, retinal ganglion cells increase expression of pro-apoptotic genes such as bax and downregulate anti-apoptotic genes as glaucoma progresses (Libby et al. 2005a). Induction of apoptosis via calcineurin cleavage and signaling has also been described as a mechanism of RGC loss in the DBA/2J and rat ocular hypertension models (Huang et al. 2005). Complement levels also remain high as the DBA/2J mouse ages and develops severe glaucoma (Stevens et al. 2007). At this stage, the complement cascade is thought to play its traditional role of opsonizing and removing the cellular debris from widespread RGC death. However, recent evidence suggests C1q may also have a neuroprotective role (Pisalyaput and Tenner 2008).

5 Role of Glial Activation in Glaucoma

Since the appearance of reactive astrocytes is an early step in the progression of glaucoma, clues to the cellular and molecular mechanisms of the disease may be found through further study of glia in the normal CNS. Glia, such as astrocytes and microglia, were once considered to be nothing more than connective tissue holding the brain together and physically keeping neurons in place. It is now clear that glia are critical participants in every major aspect of brain development, function, and disease (Barres 2008). Glial cells actively communicate with neurons, blood vessels and other glial cells to control the development and function of axons, synapses, and blood vessels (Haydon 2001; Barres 2008; Allen and Barres 2009). For example, astrocytes are present and secrete many signaling molecules during postnatal synapse development. Astrocytic processes envelop developing synapses, and are key mediators in synapse development and plasticity (Haydon 2001; Bolton and Eroglu 2009). Several astrocyte-derived synaptogenic and plasticity signals have been identified including thrombospondins, cholesterol, ApoE, and TNF-α (Mauch et al. 2001; Christopherson et al. 2005; Stellwagen and Malenka 2006).

Astrocytes also secrete signals that can promote synapse elimination in the developing brain. These glial cells secrete a factor that can trigger neuronal upregulation of C1q, the initiating protein of the classical complement cascade. Microglia are also a major source of C1q in the brain. As C1q and downstream C3 localize to synapses in the postnatal brain and retina and are required for synapse elimination (Stevens et al. 2007), identification of the astrocyte-derived signal that upregulates C1q will be important to studying the role for astrocytes in synapse elimination in vivo during development and disease. Interestingly, recent gene profiling of both immature and reactive astrocytes has shown these cells to express a similar array of genes and signaling pathways (Cahoy et al. 2008). Since synapse development and circuit

Table 2 Progression of known pathological events in glaucoma

Normal vision	Detectable vision loss	Increased vision loss	Substantial vision loss/Blindness
Preclinical	Early stage	Mid-stage	Late stage
• Elevated IOP • Vasculature abnormalities	• Astrocyte activation • Complement upregulation • Synapse loss? • RGC gene expression changes	• Astrocyte activation • Complement upregulation • Microglial activation • Synapse loss • Excitotoxicity? • Increased inflammatory cytokines • BBB disruption • RGC apoptosis	• Astrocyte activation • Complement upregulation • Microglial activation • Abnormal APP cleavage • Excitotoxicity? • Widespread RGC loss

maturation are correlated with the arrival of immature astrocytes and their association with developing synapses, the early appearance of reactive glia (Table 2) in neurodegenerative diseases suggests that reactive glia may serve as a trigger promoting glaucoma pathogenesis.

In addition to their roles in development and normal brain function, glia serve as part of the immune system of the brain and are the first to respond to damage or infection. Glial activation and proliferation during glaucoma has been described in several animal models of glaucoma, as well as in human glaucomatous eyes. In experimentally induced glaucoma, astroglial activation can be observed as early as 2 h after raising IOP levels, before widespread apoptosis of retinal ganglion cells (Woldemussie et al. 2004; Kanamori et al. 2005) (Fig. 1). Astrocyte activation has been shown in the retina and in the downstream LGN and visual cortex in primates after optic nerve transection or ocular hypertension induction before RGC degeneration (Lam et al. 2009). In cases of moderate to severe glaucoma, large clusters of reactive microglia are present in the compressed lamina cribrosa and as formations of concentric circles surrounding blood vessels (Neufeld 1999). Reactive glia can be identified by well-described markers including the upregulation of proteins involved in forming the intermediate filament network including GFAP, vimentin, and nestin in astrocytes (or Müller cells in the retina) and the expression of CD68, a lysosome-associated glycoprotein, and HLA-DR (MHC class II) in microglia (Fig. 1) (Pekny and Nilsson 2005). Activated microglia also upregulate phagocytic and chemotactic receptors such as CR3/CD11b (Lynch 2009), as well as complement. Both microglia and astrocytes exhibit morphological changes corresponding to the gene expression changes described above. Resting microglia in the CNS have a highly ramified, finely branched morphology with little cytoplasm. As these cells

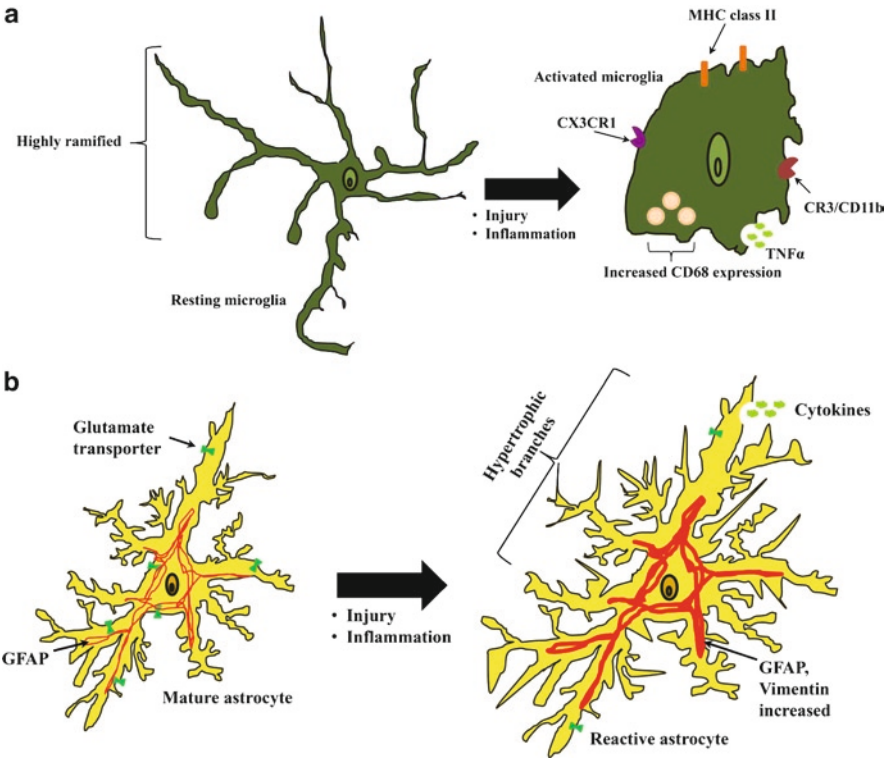

Fig. 1 Known markers of glial activation. Glial activation and proliferation is thought to contribute to the pathogenesis of glaucoma. (**a**) Microglia proliferate and are activated in mid to late glaucoma. Activation via cytokines causes microglia to adopt an amoeboid morphology and express phagocytic receptors (CR3/CD11b), MHC class II complexes, cytokines, and chemotactic receptors. (**b**) Astrocyte activation is marked by increased expression of GFAP, vimentin, and nestin as well as hypertrophy of processes. Reactive astrocytes downregulate glutamate transporters (i.e. GLAST) and release an array of cytokines and inflammatory molecules

become reactive and begin actively phagocytosing material, they adopt a bushy amoeboid morphology (Fig. 1) (Lynch 2009). Similarly, mature, resting astrocytes have a bushy appearance due to their many fine processes, while reactive astrocytes exhibit highly branched, hypertrophic morphologies and have an enhanced intermediate filament network in the soma and main processes (Pekny and Nilsson 2005).

Recent work has shown that all three major types of glia in the central nervous system (astrocytes, microglia, and oligodendrocytes) are involved in the progression of glaucoma. Astrocytes form a meshwork through which RGC axons must pass as they leave the eye. Activation of astrocytes in this region has been observed in the DBA/2J model, as well as in other rat and primate models of glaucoma induced by high IOP (Howell et al. 2007a). Whether astrocyte activation is a direct result of IOP elevation or a factor contributing to high IOP is unknown. It has been suggested that activation of astrocytes in the lamina cribrosa causes an initial insult to RGC axons, causing Wallerian degeneration of RGCs (Howell et al. 2007a; Daniel et al. 2009).

Reactive astrocytes may also contribute to the pathogenesis of glaucoma through failure to maintain neuronal networks. One of the main roles of astrocytes in the nervous system is to clear excess glutamate from synapses via glutamate transporters such as GLAST and GLT1 (Rothstein et al. 1996). In reactive astrocytes, glutamate transporters become downregulated, diminishing the cell's capacity for glutamate removal (Harada et al. 2007). Without the help of astrocytes to clear glutamate from the retina, RGCs become susceptible to glutamate toxicity or excitotoxicity. Consistent with this hypothesis, a downregulation of glutamate transporters and receptors has been observed in neurons of glaucomatous eyes and treatment with glutamate antagonists can be neuroprotective and slow RGC degeneration (Naskar et al. 2000).

Microglia, the primary phagocytic cells of the brain, also are activated in glaucoma. These cells proliferate significantly and become activated in moderate to late stages of glaucoma. Microglial activation is thought to be a major contributor to neuronal death. Specifically, the patchy pattern of neuronal death observed in late stages of glaucoma. In support of this hypothesis, addition of minocycline, which inactivates microglia, or deletion of CD11b, a phagocytic receptor on microglia, has been shown to have neuroprotective effects, delaying RGC death and axon loss in several experimental and genetic models of glaucoma (Shimazawa et al. 2005; Levkovitch-Verbin et al. 2006; Nakazawa et al. 2006; Bosco et al. 2008). One way microglia trigger RGC death is through release of pro-apoptotic cytokines. Microglia are a major source of cytokines in the brain. Microglia can initiate apoptosis of RGCs through cytokine signaling pathways, such as the tumor necrosis factor alpha pathway (TNF-α). TNF-α specifically becomes upregulated in the early stages of glaucoma and activated microglia are the most likely source of this cytokine (Tezel et al. 2008). Recent work demonstrated that injecting TNF-α intraocularly is sufficient to trigger glaucomatous symptoms and injecting TNF-α neutralizing antibodies in ocular hypertensive rats is neuroprotective (Nakazawa et al. 2006). Microglia can also serve a neuroprotective role, however, particularly when the blood-retinal barrier becomes damaged. Microglia clear debris and have also been shown to secrete factors that promote RGC survival and regeneration (Neufeld 1999; Leon et al. 2000).

Loss of oligodendrocytes, the myelinating glia of the CNS, via TNF-α apoptotic signaling is thought to contribute to RGC degeneration as well (Nakazawa et al. 2006). The degeneration resulting from the loss of oligodendrocytes can be prevented by adding oligodendrocyte precursor cells (OPCs) to the glaucomatous eye or by blocking TNF-α signaling (Nakazawa et al. 2006; Bull et al. 2009). OPCs have not been shown to re-myelinate degenerating axons, but rather secrete RGC survival factors in the presence of inflammatory cells (Bull et al. 2009). The dramatic neuroprotective effects of OPCs support further investigation into potential stem cell therapies for glaucoma.

6 Neural-Immune and Neuron-Glia Signaling in Glaucoma

Glial cells pleiotropically signal with each other and RGCs via release of cytokines and other inflammatory signaling molecules. The array of cytokines glia secrete could promote further inflammation. These inflammatory cues can cause the blood brain

barrier to become leaky, allowing inflammatory cells from the bloodstream to invade the eye (Janzer and Raff 1987; Yang et al. 1999). As mentioned above, TNF-α expression in particular has been implicated in glaucoma. This multifunctional cytokine can induce RGC death through receptor-mediated caspase activation, mitochondrial dysfunction, and oxidative stress (Tezel et al. 2008). In addition to direct neurotoxicity, potential interplay of TNF-α signaling with other cellular events associated with glaucomatous neurodegeneration may also contribute to spreading neuronal damage by secondary degeneration. Opposing these cell death-promoting signals, binding of TNF receptors can also trigger the activation of survival signals via induction of heat shock proteins and NF-κB signaling (Nakano et al. 1996). A critical balance between a variety of intracellular signaling pathways may determine the predominant in vivo bioactivity of TNF-α as best exemplified by differential responses of RGCs and glia to TNF-α signaling.

The expression of other cytokines and growth factors such as the TGF-βs, ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF) and the receptors for these factors can trigger astrocytes to adopt a reactive phenotype which can cause further inflammation and promote degeneration as described above (Pekny and Nilsson 2005). In addition, reactive astrocytes present around the optic nerve head in glaucomatous eyes express nitric oxide synthases (NOS) (Zhong et al. 2007). Normal, non-glaucomatous astrocytes do not express high levels of this enzyme. Excessive production of nitric oxide can cause death in a variety of cell types including RGCs; however, a mechanistic connection between human glaucoma and nitric oxide has not been established.

Another interesting change in neurons during glaucoma is abnormal APP cleavage. The same toxic amyloid-beta cleavage products present in Alzheimer's disease appear in both human glaucoma and experimental models (McKinnon et al. 2002; Yoneda et al. 2005). RGCs undergoing apoptosis exhibit activation of caspase-3, which is known to cleave amyloid precursor protein (APP) producing the toxic amyloid-beta fragments which have been implicated in Alzheimer's disease (McKinnon 2003). The production of amyloid-beta in glaucoma suggests another hypothetical pathway promoting RGC death via amyloid-beta toxicity and a recent study showed that inhibiting amyloid-beta aggregation and production reduced levels of RGC apoptosis (Guo et al. 2007).

7 Complement Cascade Upregulation and Activation in Glaucoma

Concurrently with glial activation, complement gene expression and protein levels have been shown to rise significantly in the glaucomatous eye. In microarray studies from retinas of DBA/2J mice, experimental rat and primate models, and human glaucoma, both C1q and downstream components (C3, C4) are highly expressed early in the disease long before RGC death (Ahmed et al. 2004; Kim et al. 2005; Kuehn et al. 2006; Stasi et al. 2006; Steele et al. 2006; Stevens et al. 2007). Recent work has shown C1q localizes to synapses in the inner plexiform layer (IPL) of the DBA/2J retina concurrent with a decrease in synapse density (Stevens et al. 2007). In contrast

to genetically matched control mice, punctate C1q immunoreactivity was present in the synaptic IPL in retinas from aged DBA/2J mice. Importantly, C1q localized to synapses in eyes that were determined to have "no or early" symptoms of glaucoma, and have no detectable RGC loss. There was a more pronounced increase in C1q synaptic staining in the IPL of retinas from eyes with moderate (and severe) glaucoma that corresponded to a decrease in synapses and RGC loss. Interestingly, a recent study investigating ischemia-reperfusion in the eye has shown that mice deficient in C3 show reduced optic nerve damage and RGC degeneration 1 week after ischemia, (Kuehn et al. 2008). Together, these findings support a role for complement in the early stages of retinal degeneration and suggest that complement-mediated synapse loss occurs as a crucial early event in glaucoma.

Aberrant complement deposition at the synapse is a potential mechanism for the synapse loss seen in early stages of glaucoma and other neurodegenerative diseases; however, recent studies also have shown that C1q can have a neuroprotective role. In Alzheimer's disease (AD) models, inhibition of C3 and other early components of the cascade resulted in increased pathology (Wyss-Coray et al. 2002; Maier et al. 2008). One theory to explain this neuroprotective effect is that complement components are essential for the clearance of amyloid plaques that occur in Alzheimer's disease and other cellular debris that can be cytotoxic. Further, the addition of C1q to primary neuronal cultures has been shown to be neuroprotective against caspase and calpain mediated neurotoxicity which are prominent in glaucoma (Pisalyaput and Tenner 2008). These results both suggest that the environment in which complement component expression occurs may be important in determining if these proteins have a protective or harmful effect on the cells. Uncleaved, inactivated components could potentially promote survival of the cell via novel signaling pathways. Conversely, C1q may play more of a neuroprotective role if the downstream substrates for the C1 complex are not expressed.

The events thought to trigger RGC loss in glaucoma are also observed in other neurodegenerative diseases. As mentioned above, amyloid-beta (Aβ) production occurs in both Alzheimer's (AD) and glaucoma, but these disorders, along with amyotrophic lateral sclerosis (ALS) and Parkinson's disease, also exhibit complement upregulation activation, synapse loss, and glial activation (Sasaki and Maruyama 1994; Hirsch et al. 2003; Turner et al. 2004; Lobsiger et al. 2007; Alexander et al. 2008; Chiu et al. 2008, 2009). The universality of these processes suggests a common mechanism in these diseases underlying neuronal dysfunction and death.

8 Parallels Between Complement-Mediated Synapse Elimination and Synapse Loss

Emerging evidence suggests that synapse loss precedes RGC death in glaucoma. In a rat model of glaucoma, loss of connectivity between RGCs and lateral geniculate neurons is seen as early as 5 days after elevation of IOP (Fu et al. 2009). Similarly, in

non-human primate models of glaucoma, alterations in the visual fields and the lateral geniculate nucleus are observed 2–3 weeks after IOP increase through photocoagulation (Sasaoka et al. 2008). In the DBA/2J mouse model, a decrease in synapse density is observed prior to RGC loss (Stevens et al. 2007). Together these results suggest that synapse loss may be one of the early events in the progression of glaucoma. Thus, understanding how immature synapses are eliminated during development in the normal brain may provide insight into how mature synapses may be aberrantly targeted for destruction in glaucoma and other neurodegenerative diseases.

The first 2 weeks of postnatal development is a critical period in CNS development during which synapses are formed and refined (Katz and Shatz 1996; Hua and Smith 2004; Huberman et al. 2008). During this period immature astrocytes associate with synapses and secrete many cytokines and other signaling molecules some of which have been implicated in synapse formation and synaptic plasticity (Ullian et al. 2001; Ullian et al. 2004; Barres 2008). A screen to determine how astrocytes influence neuronal gene expression first identified C1q as one of the few genes highly upregulated in developing RGCs in response to an astrocyte-derived secreted factor (Stevens et al. 2007). This was a surprising finding, as C1q was not thought to be expressed in the healthy brain. Importantly, C1q, and downstream complement protein, C3, were found highly expressed in synaptic regions of the developing retina and brain and co-localized with synaptic markers (Stevens et al. 2007). Given the well-described role of complement in the innate immune system, these findings suggested that the complement cascade may be opsonizing or "tagging" inappropriate synapses for elimination in the developing brain. Consistent with this hypothesis, neuroanatomical tracings of retinogeniculate projections and electrophysiological recordings in LGN neurons in C1q KO and C3 KO mice showed sustained defects in synapse elimination (Stevens et al. 2007).

How are synapses eliminated by complement? One hypothesis is that microglia phagocytose complement-"tagged" synapses. In the immune system, peripheral phagocytic cells (e.g. macrophages) rapidly clear complement-opsonized cells and debris via high affinity complement receptors such as complement receptor 3 (CR3). In support of this hypothesis, microglia are the only resident CNS cell to express CR3 (Rotshenker 2003). Microglia also secrete C1q and most complement cascade components, and activated or "reactive" microglia localize to the grey matter of cerebellum, hippocampus, and other brain regions during a narrow window of postnatal development that coincides with the peak of synapse formation and elimination (Milligan et al. 1991; Dalmau et al. 1998; Maslinska et al. 1998; Fiske and Brunjes 2000).

Synapse elimination is an activity-dependent process (Torborg and Feller 2005), but surprisingly the molecular mechanisms linking neural activity with the physical elimination of a synapse are unknown. One model of synapse elimination proposes that strong synapses, which are effective in driving postsynaptic responses, actively punish and eliminate nearby weaker synapses by inducing two activity-dependent signals: a short-range "protective" signal and a longer range "punishment" signal (Jennings 1994). The identities of these signals remain elusive; however, components of the complement cascade and the regulatory proteins controlling the

cascade are well-suited to act as these punishment and protective factors because of their traditional roles in the immune system. The opsonins in the complement cascade, such as C1q and C3b, are hypothesized to be the "punishment" signals in this model. What could prevent aberrant elimination of appropriate mature synapses? One hypothesis for how this protection is achieved is through activity dependent expression of complement regulatory proteins at synapses. These regulatory proteins, which include molecules such as CR1, DAF, MCP, and CD59, prevent complement deposition or activation and are expressed at the cell membrane (Griffiths et al. 2007; Zipfel and Skerka 2009). Interestingly, complement regulatory proteins have been shown to be expressed at lower levels in developing neurons than in other cells of the CNS (Singhrao et al. 1999). There are two possible scenarios by which complement regulatory proteins could direct synapse elimination. One hypothesis is that C1q initially "tags" most synapses and its activation is prevented at strong synapses by activity-dependent expression of complement regulatory proteins. Alternatively, complement regulatory proteins prevent deposition of complement at strong synapses such that only weak synapses are selectively "tagged". Thus, understanding which synapses are targeted by complement and whether electrical activity is required for activation of the complement cascade are important questions for future investigation.

9 Conclusions and Perspectives

The similarities between developmental synaptic pruning and the early stages of synapse loss in neurodegenerative disease suggest a model in which immature or reactive astrocytes trigger neuronal upregulation of C1q (Fig. 2). Binding of C1q at synapses activates downstream complement cascade components, including C3, which also binds synapses. Activated microglia are recruited to the brain's synaptic regions where complement upregulation is occurring, and eliminate complement (C1q and/or C3b) "tagged" synapses via phagocytic mechanisms. Alternatively, complement can act in the absence of microglial phagocytosis to trigger synapse loss by activation of downstream membrane attack complex (MAC) or other mechanisms.

If synapse loss in the diseased brain is mediated by the same mechanisms as developmental synapse elimination, why do neurons degenerate in the diseased brain but not during development? One possibility is that complement expression is limited in the developing brain by the maturation of astrocytes. When astrocytes reach their mature phenotype, they may stop secreting the signal to promote high levels of C1q transcription. In the diseased brain, reactive glia may continue to upregulate and produce complement components and thus could cause neuronal loss and damage. Prolonged glial activation could also increase the probability of deposition of the membrane attack complex, which has been implicated in neurodegenerative disease but not in normal development (Webster et al. 1997). A reduction in complement regulatory proteins in neurons such as CD59 has been shown in Alzheimer's disease concurrent with an increase in MAC levels (Yang et al.

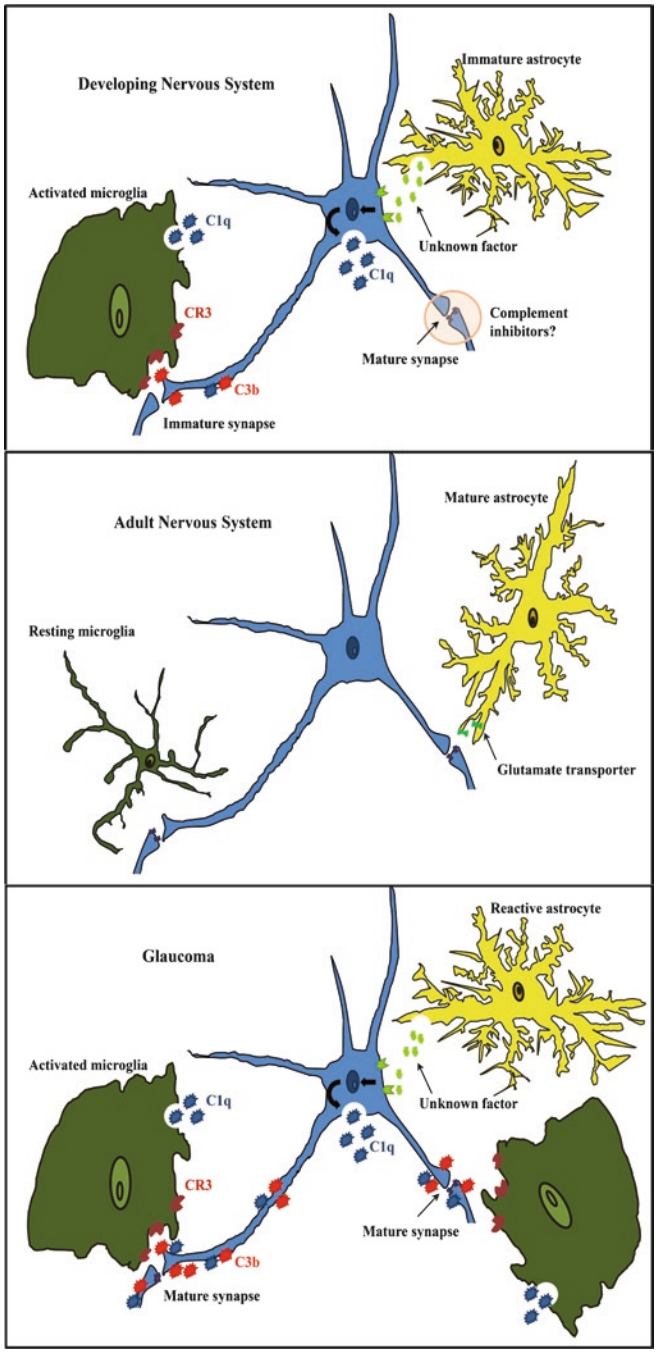

Fig. 2 Parallels between developmental synapse elimination and synapse loss in glaucoma. In the developing visual system, complement is hypothesized to be upregulated in neurons by unknown signals from immature astrocytes. C1q and downstream C3b localize to synapses, opsonizing them for elimination by phagocytic microglia. Weak synapses are thought to be protected from elimination

2000). As these proteins normally protect neurons from complement deposition, a deficit in CD59 or other complement regulators may contribute to neuronal degeneration as well. Adult neurons also simply may lack the ability to recover from synapse loss. Immature neurons are known to exhibit a higher degree of plasticity and are pruned prior to myelination (Huberman et al. 2008). The adult CNS is a less plastic environment with numerous cues that inhibit regeneration and the formation of new circuitry.

If similar mechanisms mediate both developmental synapse elimination and synapse loss during disease, then the molecules mediating developmental synapse elimination may provide therapeutic insight for neurodegenerative disorders. For example, identification of the signal that triggers C1q upregulation in RGCs could uncover potential targets for early therapeutic intervention to prevent synapse and RGC loss in glaucoma. In addition, endogenous complement regulatory proteins or synthetic complement inhibitors are potential classes of molecules that can be useful therapeutically (Sahu and Lambris 2000; Carroll 2004; Ricklin and Lambris 2008). Endogenous complement regulatory and inhibitory proteins are highly expressed in the CNS (Griffiths et al. 2007). Defects in endogenous inhibitors have been linked to auto-immune disorders such as lupus (Nagy et al. 1999) and recently a genetic link between Factor H and macular degeneration has been established (Hageman et al. 2005). In addition to utilizing endogenous complement inhibitors to control complement activity, several synthetic inhibitors such as compstatin (Ricklin and Lambris 2008) show promise for the treatment of the many pathological conditions exhibiting complement activation. A complicating factor for treating glaucoma with complement inhibitor proteins is that complement also could be a neuroprotective factor at certain stages of the diseases in addition to its potential role as a glaucoma "initiator" (Pisalyaput and Andrea 2008). Further investigation is necessary to determine at which stages and at what levels glial activation and complement expression are beneficial or harmful.

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Fig. 2 (continued) by complement regulatory proteins. In the healthy adult brain, glial cells are quiescent. Microglia downregulate phagocytic receptors and inflammatory cytokines. Astrocytes support synaptic function. In glaucoma and other neurodegenerative diseases, the mechanisms that trigger developmental synapse elimination are thought to be re-activated and aberrantly eliminate synapses.

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Chapter 7
A Role for Complement in Glaucoma?

Lizhen Ren and John Danias

Abstract Chronic open angle glaucoma is a degenerative optic neuropathy that can lead to blindness. We have shown that one of the major genes with altered expression in the glaucomatous retina is complement component C1q in both animal models of the disease as well as in humans. These observations together with evidence of upregulation of other complement components within the retina suggest a role for complement in the pathogenesis of this disease. We review the current evidence that supports such a role and discuss possible mechanisms through which complement may act. A thorough understanding of these mechanisms is important in allowing us to rationally design new therapeutic approaches.

1 Introduction

Glaucoma is a potentially blinding neurodegenerative disease. In reality, glaucoma is a family of diseases with similar characteristics that should be referred to as "the glaucomas". Glaucomas account for approximately 15% of all blindness worldwide (Thylefors and Negrel 1994) and are collectively one of the three leading causes of blindness in the United States (Coleman and Brigatti 2001). They are a family of progressive optic neuropathies with characteristic optic nerve head appearance that differentiates them from all other optic neuropathies. Because of the anatomic arrangement of the nerve fibers within the retina and optic nerve, glaucomas also manifest characteristic peripheral vision defects and are often associated with high intraocular pressure (IOP). Two clinically distinguishable forms of adult-onset glaucoma exist: Open-angle glaucoma (OAG), where the angle of the anterior chamber is anatomically open, and angle-closure glaucoma where this angle is anatomically closed .The angle of the anterior chamber of the eye is formed at the

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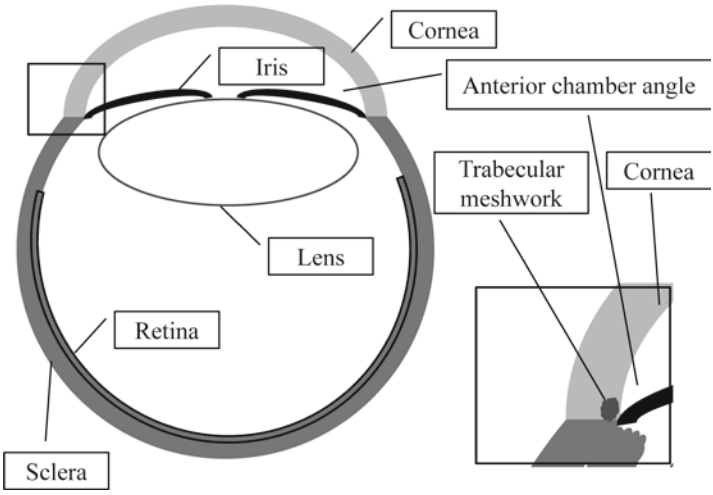

Fig. 1 Schematic of the human eye. *Inset* shows the location of the trabecular meshwork

intersection of the iris root with the corneal periphery (see Fig. 1). It is the area through which aqueous humor is drained and is thus important in glaucoma as closure of the angle can lead to high IOP. Intraocular pressure regulation depends on a balance between aqueous humor production and aqueous humor elimination, in the front of the eye, in the areas of the trabecular meshwork and surrounding tissues (Fig. 1). Most of the glaucoma encountered in the western world is of the open-angle type, while the incidence of angle closure glaucoma is much higher in the Far East (Cedrone et al. 2008). This review is focused mostly on the relationship of complement to OAG.

Open-angle glaucoma is a slowly progressive disease. Based on information from a number of long-term studies OAG leads to blindness in approximately 20% of patients over a period of 20 years (Oliver et al. 2002; Wilson et al. 2002). It affects predominantly older individuals, with yearly incidence of the disease increasing from less than 0.3% at ages 40–49 to ~0.8% above age 70 (Leske et al. 2007b). The prevalence of the disease varies between 1 and 16% in various populations (Rudnicka et al. 2006) with African-Americans being disproportionately affected (Tielsch et al. 1991). Although elevated intraocular pressure is often associated with the disease, 20–50% of the patients with what is considered to be intraocular pressure within the normal range develop characteristic glaucomatous optic neuropathy and are often referred to as having normal or low-tension glaucoma (Sommer et al. 1991; Shields 2008). Intraocular pressure, however, has been one of the few risk factors for the development (Gordon et al. 2002) and progression (Leske et al. 2003, 2007a) of the disease that has been identified to date (Nemesure et al. 2007) and the only one that is modifiable (Leske 2007). IOP reduction forms the basis for our current therapy of glaucoma. A number of multicenter studies (Gordon et al. 2002; Beck 2003; Leske et al. 2003; Parrish et al. 2009) have shown

that intraocular pressure decrease leads to significant reduction in the rate of glaucomatous optic nerve neuropathy, even in patients with normal tension glaucoma (Anderson 2003).

Despite improvements in our understanding of many of the factors involved in regulation of intraocular pressure, we still do not understand the neurodegenerative aspect of glaucoma. Most of the available evidence points to a process that initiates at the level of the optic nerve head (Downs et al. 2007; Howell et al. 2007a; Yang et al. 2007a, b). The initial damage at this level seems to propagate both retrogradely as well as anterogradely. Ultimately it leads to a demise of the retinal ganglion cell bodies that reside within the retina and whose axons make up the optic nerve (Quigley 1995). At the same time, the decentralized axons, that form the part of the optic nerve also degenerate, leading to secondary degeneration of CNS structures (Yucel et al. 2000; Gupta et al. 2007, 2009; Yucel and Gupta 2008). Some evidence also suggest that this neurodegeneration may occur in two phases; a primary phase initiated by an initial insult and a secondary phase (Levkovitch-Verbin et al. 2001) that occurs independent of intraocular pressure and has been attributed to toxic factors (Wax and Tezel 2002) released by dying ganglion cells or their axons or malfunctioning glia at either the level of the optic nerve or the retina (Wax and Tezel 2002).

Retinal ganglion cells die by apoptosis (Kerrigan et al. 1997; Cordeiro et al. 2004) and thus do not elicit significant inflammatory response. The absence of an inflammatory response was actually considered one of the distinguishing features of glaucoma until a few years ago when a number of investigators started questioning whether components of the inflammatory system may actually be involved in this process (Tezel et al. 1998; Wax 2000). Recent work has implicated lymphocytes (Schwartz 2004, 2005; Schwartz and Kipnis 2005), as well as resident microglia (Tezel 2009) and dendritic cells (Schwartz 2007) in the retina and the optic nerve (Tezel et al. 2007) in the pathogenesis of the disease. However, much of this work has been performed in animal models of the disease and human data are sparse. It becomes thus important to look critically at the animal models used to generate these data. The primate model of glaucoma, which is developed by lasering the trabecular meshwork (Pederson and Gaasterland 1984; Rasmussen and Kaufman 2005) and thus increasing intraocular pressure, is the one most closely related to the human disease (Rasmussen and Kaufman 2005). Much of the data that has been generated recently however, relies on development of glaucoma models in rodents. Glaucoma models in rats (Weinreb and Lindsey 2005) involve increase in intraocular pressure; using a variety of methods to obstruct the outflow pathways either through sclerosing the episcleral veins with hypertonic saline (Johnson et al. 1996; Morrison et al. 1997) or by cauterization (Shareef et al. 1995; Mittag et al. 2000; Bayer et al. 2001) or directly injecting microspheres or particles in the anterior chamber (Urcola et al. 2006). The most widely used mouse glaucoma model is the DBA/2J mouse strain (Sheldon et al. 1995; John et al. 1998; Danias et al. 2003). These mice, develop a spontaneous IOP elevation starting after approximately the fourth month of age and a progressive optic nerve and retinal ganglion cell degeneration starting after the eighth month of age (Libby et al. 2005a). The glaucoma like

pathology in these animals appears to share a lot of common characteristics with the human disease including the "patchy" or regional distribution of damage (Danias et al. 2003; Jakobs et al. 2005), the initiation of damage at or around the optic nerve head (Jakobs et al. 2005; Howell et al. 2007a) and the relation between intraocular pressure and the amount of damage (Libby et al. 2005b). In addition, despite the DBA/2J animals being an inbred strain, there is significant variability between different animals and the amount of damage they sustain, and occasionally even between the two eyes of a particular animal (Danias et al. 2003; Schlamp et al. 2006). It has been shown that mutations in the Tyrp-1 and GPNMB genes are causative (Anderson et al. 2002), but either one alone not sufficient to generate the pathology observed in the DBA/2 animals (Howell et al. 2007b). Introduction of both of these mutations into the C57BL/6 strain failed to replicate the intraocular pressure as well as retinal and axonal loss phenotype (Anderson et al. 2006). Of course, despite similarities, mouse optic nerve head anatomy is not identical to that of humans (Howell et al. 2007a) and thus extrapolations from this animal model have to be performed cautiously.

2 Complement and Glaucoma

Involvement of complement in the pathogenesis of glaucoma was initially reported in microarray experiments performed in 2003 on primate eyes (Miyahara et al. 2003). However, the potential significance of these findings was not appreciated. A more detailed evaluation of initiation components of the complement in glaucoma was performed in 2006 (Stasi et al. 2006). It was shown that in DBA/2 mice, C1q is specifically up-regulated in the retina starting early in the course of the disease (at around the time IOP begins to rise) and progressively increases as glaucomatous neurodegeneration progresses. In fact C1q up-regulation occurs before any appreciable damage can be detected in the retina, suggesting that C1q up-regulation may be a component that leads to retinal ganglion cell loss (Stasi et al. 2006). These initial findings were confirmed in both primate and human glaucoma specimens (Stasi et al. 2006) and replicated in the rat glaucoma model of induced ocular hypertension (Kuehn et al. 2006) and in human specimens (Kuehn et al. 2006). They were also extended by showing that C3 is also up-regulated in the retinal ganglion cells in the rat glaucoma model and human eyes (Kuehn et al. 2006) and that RGCs in culture could be induced to change expression of complement components by serum deprivation (Khalyfa et al. 2007).

Although, C1q and C3 appear to be locally synthesized in the retina, it is yet unclear which cells within the retina produce these complement components, as localization of the proteins on a certain cell does not prove that these complement components are actually synthesized by these cells. Localization of synthesis is important to generate plausible hypotheses about the function of these molecules. Some answers to these questions were provided by the work of Stevens et al. (2007) who reported that C1q up-regulation in cortical neurons leads to synapse elimination.

These investigators also showed that C1q knockout as well as C3 knockout animals failed to make the necessary anatomical refinements of the retinogeniculate connections causing the retention of excess retinal innervation by lateral geniculate neurons. They also reported that in the DBA/2 mouse, C1q and C3 up-regulation occurs before damage develops and localizes mostly at the inner plexiform layer (the layer where the ganglion cells synapse with the amacrine, bipolar and horizontal cells). This finding suggested that C1q may actually play a role in the early course of glaucoma by pruning the dendritic fields of ganglion cells leading these to later become apoptotic. In later stages of the disease, C1q appeared to localize at the Muller cell glia [as reported by others (Stasi et al. 2006)] within the retina (personal communication), however, the finding was not consistent.

So what is complement doing in glaucoma? It is well known that complement is present in the eye at very low concentrations and that most if not all complement components are synthesized locally (Sohn et al. 2000; Jha et al. 2006). This makes sense, since both in the anterior part of the eye as well as within the retina, there exists a barrier that excludes large proteins like complement components entering the aqueous and retina respectively. These low levels of complement proteins thus help protect this immunologically privileged tissue and appear to be at a low constant level of activation (Sohn et al. 2000). One can of course speculate that inappropriate complement activation could directly lead to ganglion cell loss in glaucoma through activation of the membrane attack complex (Muller-Eberhard 1986). However, this is unlikely for two main reasons:

1. MAC activation (at least at lytic levels) tends to be rather messy in the way it kills cells and is often accompanied by local infiltration by macrophages (Lautenschlager et al. 1999; Xiong et al. 2003; Bohana-Kashtan et al. 2004) or other immune cells that need to clean up the debris created from disintegration of the dying cell. It is specifically this lack of massive immune cell infiltration that has to date made glaucoma be considered a non immune-related disease.
2. DBA/2 mice, which develop a pathology that in many respects resembles human glaucoma, are C5 deficient and thus cannot activate MAC (Pasinetti et al. 1996). This of course begs the question of whether C1q up-regulation in these animals leads to RGC loss through alternate pathways and what these may be.

One additional question regarding the role of C1q is whether up-regulation and subsequent dendritic pruning that it causes are destructive or rather protective of RGCs within the retina. It has been suggested (Villalba and Navas 2000) that a decrease in electrical activation of neurons in stress may actually be a protective mechanism in an effort to avoid undergoing programmed cell death. If that is the case for RGCs, early changes in complement activation in glaucoma may be protective.

But how is then the fact that complement activation increases with progression of the disease explained at least in the DBA/2J mice? One would expect that continued loss of retinal ganglion cells would decrease the amount of C1q and thus complement activation as the disease progresses. That is not seen though at least in the DBA/2 mouse model. The apparent shift in immunostaining from RGCs to glial

elements within the retina is also hard to explain. As was earlier pointed out, immunostaining early on appears to concentrate at the IPL (Stevens et al. 2007) while later on it is mostly associated with Muller glia and astrocytes (Stasi et al. 2006). One possible way to explain this finding would be that complement may have varied roles depending on the stage of the disease and on the cells on which it is expressed. Early on in the process of the disease complement activation may play a positive (protective) role decreasing the size of the dendritic trees and thus lowering electrical activation of retinal ganglion cells in an effort to avoid the death. If death is inevitable, and apoptosis occurs, then complement may actually opsonize debris from apoptotic cells and help in their clearance, thus significantly decreasing the inflammatory response (Mevorach 2000; Kim et al. 2003; Gaipl et al. 2006; Gullstrand et al. 2009).

Such dual role for complement is actually very appealing as it would explain some of the peculiarities seen in the various glaucoma models. However, it makes development of therapeutics based on complement to prevent glaucomatous neurodegeneration rather challenging as RGC damage in glaucoma is asynchronous and the disease progresses rather slowly. In this instance blockage or enhancement of complement activation may have opposing effects on different cells within the retina at the same time.

As with other neurodegenerations, understanding the role of complement in glaucoma is not an easy task. Development of knockout animals in the DBA/2 background will provide some crucial answers as to this role. A number of labs around the world have started generating these animals and we should expect some answers emerging in the next few years. We should not be surprised if complement turns out to be much more complex than we think even at this time point.

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Chapter 8
The ATP-Binding Cassette Transporter ABCA4: Structural and Functional Properties and Role in Retinal Disease

Yaroslav Tsybovsky, Robert S. Molday, and Krzysztof Palczewski

Abstract ATP-binding cassette transporters (ABC transporters) utilize the energy of ATP hydrolysis to translocate an unusually diverse set of substrates across cellular membranes. ABCA4, also known as ABCR, is a ~250 kDa single-chain ABC transporter localized to the disk margins of vertebrate photoreceptor outer segments. It is composed of two symmetrically organized halves, each comprising six membrane-spanning helices, a large glycosylated exocytoplasmic domain located inside the disk, and a cytoplasmic domain with an ATP-binding cassette. Hundreds of mutations in ABCA4 are known to cause impaired vision and blindness such as in Stargardt disease as well as related disorders. Biochemical and animal model studies in combination with patient analyses suggest that the natural substrate of ABCA4 is retinylidene-phosphatidylethanolamine (*N*-retinylidene-PE), a precursor of potentially toxic diretinal compounds. ABCA4 prevents accumulation of *N*-retinylidene-PE inside the disks by transporting it to the cytoplasmic side of the disk membrane where it can dissociate, allowing the released all-trans-retinal to enter the visual cycle. The pathogenesis of diseases caused by mutations in ABCA4 is complex, comprising a loss-of-function component as well as photoreceptor stress caused by protein mislocalization and misfolding.

1 Introduction to ABC Transporters

ATP-binding cassette transporters (ABC transporters) utilize the energy of ATP hydrolysis to unidirectionally translocate a diverse set of substrates, ranging from ions to lipids and peptides, across cellular membranes (Higgins 1992). These ubiquitous integral membrane proteins are present in all living organisms and constitute one of the largest classes of proteins (Kos and Ford 2009; Linton and Higgins 2007). ABC transporters can function as either importers or exporters, moving their

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substrates in or out of the cytoplasm, respectively (Dawson et al. 2007). As a result, these proteins participate in a great variety of biological processes that involve active transport of substances across extracellular or intracellular membranes, for example, in nutrient uptake and drug resistance.

Despite a generally low sequence identity, all ABC transporters share the same architecture (Kos and Ford 2009; Linton and Higgins 2007; Rees et al. 2009). A minimum of four domains is required for functional activity: two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs), also known as ATP-binding cassettes. TMDs are responsible for binding substrate and forming the translocation path, whereas NBDs provide energy for transport by hydrolyzing ATP to ADP. Besides these four core domains, some ABC transporters have additional elements that may be fused to TMDs or NBDs, for instance, extracellular domains located across the membrane from NBDs. The quaternary organization of ABC transporters varies. In prokaryotes, different domains are often expressed as separate polypeptide chains that associate to form a functional transporter, whereas many eukaryotic members constitute a single amino acid sequence (full transporters) or comprise two symmetrical polypeptide chains (half transporters).

2 Human ABC Transporters

To date, 49 ABC transporters have been identified in the human genome. These are organized into seven subfamilies (ABCA to ABCG) based on gene structure, amino acid sequence and phylogenetic analysis (Vasiliou et al. 2009). Subfamily A is composed of 12 proteins. Although the substrates for most of its members have yet to be identified, indirect evidence indicates that these proteins are likely to be involved in lipid transport in different organs and cell types. All members of this subfamily are large full transporters organized in two topologically similar halves and ranging from 1,543 (ABCA10) to 5,058 (ABCA13) residues in size. A distinctive feature of family A members is the presence of a large extracellular domain in the N-terminal half of the sequence. Mutations in several ABCA genes have been linked to inherited diseases such as Tangier disease (defects in ABCA1) (Zarubica et al. 2007) and harlequin type ichthyosis (defects in ABCA12) (Akiyama et al. 2005). The focus of this review is ABCA4, an ATP-binding cassette transporter predominantly found in photoreceptor cells. Defects in the ABCA4 gene lead to Stargardt disease and may also be implicated in several other severe visual disorders.

3 ABCA4 and Vision Diseases

Stargardt disease is an autosomal recessive form of juvenile macular degeneration with an estimated prevalence of 1 in 10,000 (Paskowitz et al. 2006; Walia and Fishman 2009; Weleber 1994). It is usually diagnosed within the first two decades of

life and leads to progressive irreversible loss of central vision and delayed dark adaptation. At the histological level, degeneration of photoreceptors and the underlying retinal pigmented epithelium (RPE) occurs within and near the macula. The reason for the death of RPE cells, which are responsible for maintenance of photoreceptors and phagocytosis of their aging outer segments, is believed to be the accumulation of an age-related pigment lipofuscin containing various toxic by-products of the visual cycle (Sparrow et al. 2009). In particular, elevated levels of di-retinoid-pyridinium-ethanolamine (A2E), the ultimate product of condensation of two molecules of all-*trans*-retinal and one molecule of phosphatidylethanolamine (PE), and its precursors were found in ocular tissues from Stargardt patients (Rozet et al. 1998). Degeneration of photoreceptors seems to be secondary to the loss of the RPE.

Although A2E has been widely accepted as the major harmful component of lipofuscin, other compounds have been proposed to have toxic effects on photoreceptors and the RPE (Sparrow et al. 2009). In particular, studies of the *Abca4*$^{-/-}$*Rdh*$^{-/-}$ mice revealed that all-*trans*-retinal, a precursor of A2E, is directly involved in acute light-induced retinopathy (Maeda et al. 2009a, b). From this viewpoint, formation of A2E may in fact represent a way of detoxification of photoreceptors by reducing the concentration of free all-*trans*-retinal following an intense photobleach.

In 1997, the gene defective in Stargardt disease was identified and found to encode the ABC transporter, ABCA4 (also known as ABCR) (Allikmets et al. 1997a, b). ABCA4 mutations have also been found in several other visual disorders including fundus flavimaculatus (currently considered a form of Stargardt disease) (Rozet et al. 1998), cone-rod dystrophy (Hamel 2007), and autosomal recessive retinitis pigmentosa (Martinez-Mir et al. 1998). It has also been proposed that individuals carrying mutations in ABCA4 may have a higher risk of developing age-related macular degeneration (AMD) (Allikmets et al. 1997a; Mata et al. 2001; Rozet et al. 1998), although other studies argue against this link (Schmidt et al. 2003).

4 Molecular View of ABCA4

4.1 Primary Structure

To date, the amino acid sequences of ABCA4 from nine different species have been published. These include proteins from human, crab-eating macaque, cow, mouse, rat, dog, African and Western clawed frogs, and puffer fish. Alignment of these sequences with ClustalW2 (Larkin et al. 2007) shows high similarity among ABCA4 proteins, with identity scores ranging from 62% (human vs. puffer fish) to 97% (human vs. macaque). All these primary structures feature a number of highly conserved sequence motifs mostly located in the NBDs, which are used to identify members of the ABC transporter superfamily (Fig. 1). Specifically, the ABC transporter signature motif, 'LSGGQ', is present in both NBD1 and NBD2, although it is reduced to 'SGG' in NBD2. Nucleotide-binding domains also have Walker A and B motifs typical of ATP-processing enzymes. The Walker A motif is 'GXXXXGKT'

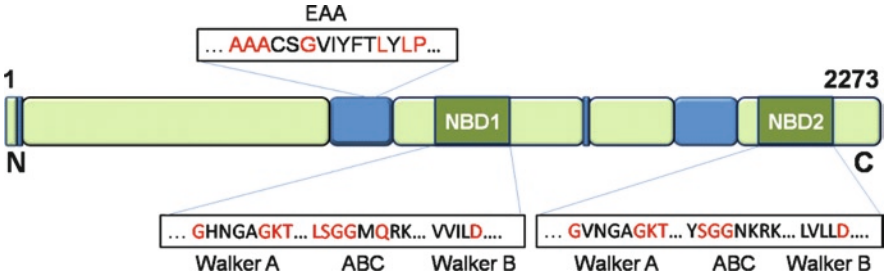

Fig.1 Diagram depicting the location of highly conserved motifs specific for ABC transporters in the primary structure of ABCA4. Predicted transmembrane regions are shown in *blue*. See text for detailed description of the various motifs

('X' is any residue) and the Walker B motif is 'hhhhD' ('h' is any hydrophobic residue). Interestingly, another ABC transporter motif, 'EAA', is located in the membrane-spanning region of the N-terminal half of ABCA4. This long motif has a sequence of 'EAAXXXGXXXXXXXIXLP', with some variations among individual proteins (Mourez et al. 1997). Importantly, it represents a signature of ABC *importers*, where it contributes to the interface between the transmembrane helices and the NBD (Rees et al. 2009). The presence of the 'EAA' motif in ABCA4 may be of interest because it is generally assumed that all eukaryotic ABC transporters are *exporters*. Curiously, the 'EAA' motif is absent in the symmetrically organized C-terminal half of ABCA4.

4.2 Localization

About 30 years ago, an electron microscopy study by Papermaster et al. (1978) of immunochemically labeled frog photoreceptors localized "a large intrinsic membrane protein" to rod outer segments. The rod outer segment (ROS) is a specialized compartment of the rod cell harboring hundreds of flattened closed membrane structures called disks, that is connected to the rest of the rod cell with a cilium (Fig. 2a). ABCA4 was shown to be situated in the rims and incisures of these disks (Fig. 2b, c). A homologous protein was later identified in bovine rod outer segments (Molday and Molday 1979) and shown to have the same localization (Illing et al. 1997). The "rim protein" was cloned and classified as a member of the ATP transporter superfamily based on its sequence homology (Allikmets et al. 1997b; Azarian and Travis 1997; Illing et al. 1997). For some time it was believed that mammalian ABCA4 was specific to rod cells (Illing et al. 1997; Sun and Nathans 1997). However, the presence of ABCA4 in foveal and peripheral cone cells was later demonstrated by immunohistochemistry and western blot analysis (Molday et al. 2000). This finding agrees well with another early study by Papermaster et al. (1982) showing that ABCA4 localized to the margins of cone outer segment lamellae in frogs.

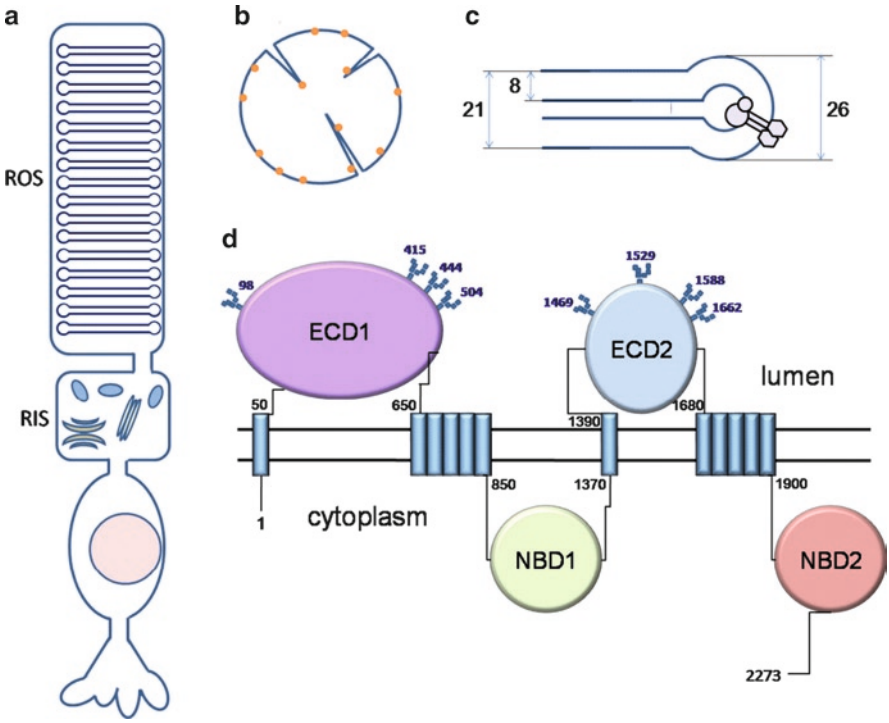

Fig. 2 Localization and topology of ABCA4. (**a**) Schematic representation of a rod cell. *ROS*: rod outer segment; *RIS*: rod inner segment. (**b**) *Top view* of a ROS disk. Molecules of ABCA4 are denoted by *yellow dots*. (**c**) *Side view* of a cross-section of a disk. ABCA4 is localized to the rim of the disk. Numbers represent dimensions of a mouse disk in nm. (**d**) Topology of human ABCA4. Known glycosylation sites are shown with *squares*. Numbers are positions of the corresponding residues in the primary structure. ECD1, ECD2: exocytoplasmic domains 1 and 2, respectively. NBD1, NBD2: nucleotide-binding domains 1 and 2, respectively

The reason for such a restricted localization of ABCA4 within the ROS disks is still unclear. A possible explanation is that the distribution of this protein is dictated by the size of its extracellular domains (also called exocytoplasmic domains 1 and 2, or ECD1 and ECD2) located in the disk lumen (Fig. 2c). Indeed, assuming a spherical shape of the larger ECD1, which comprises about 600 residues, the approximate volume of this domain calculated based on the average partial specific volumes of amino acids (Harpaz et al. 1994) is about 8 nm^3. A rough estimate of its diameter is therefore 5–6 nm, which is greater than most values for the distance between membranes of the same disk published to date (0–5 nm, depending on the species (Nickell et al. 2007)), but presumably less than the inner diameter of the rim (Fig. 2c). It was proposed that ABCA4 might play a structural role in maintaining the shape of the disk and connecting it to the plasma membrane of ROS (Roof and Heuser 1982), but this hypothesis did not receive experimental support.

So far, structural and functional studies of ABCA4 have focused on the protein expressed in the retina. But it needs to be noted that several studies detected lower

levels of ABCA4 expression in the brain (Bhongsatiern et al. 2005; Tachikawa et al. 2005). For example, the presence of ABCA4 mRNA in the lateral ventricles of the rat brain was revealed by in situ hybridization, and the expression of the protein in the choroid plexus was shown by western blotting (Bhongsatiern et al. 2005). In a more recent study, quantitative real-time PCR analysis confirmed ABCA4 mRNA expression in the brains of several mammals, including humans, with the highest level of expression observed in mice (Warren et al. 2009).

4.3 Insights into Topology, Structure and Posttranslational Modifications

Four regions containing transmembrane helices can be identified in the primary structure of ABCA4 based on hydropathy plots (Fig. 1). Two single transmembrane helices are located at approximate positions 24–46 and 1370–1392 (human protein), delineating the beginning of the N-terminal and C-terminal halves of the protein, respectively (Fig. 2d). The following exocytoplasmic domains 1 (600 residues, N-terminal half) and 2 (300 residues, C-terminal half) are situated in the disk lumen, as demonstrated by glycosylation studies (Bungert et al. 2001; Illing et al. 1997; Molday and Molday 1979). In each half of the protein, the exocytoplasmic domain is succeeded by the second hydrophobic region presumably comprising five transmembrane helices. Thus, the total number of transmembrane helices in ABCA4 is most likely 12, in agreement with what was shown for many other ABC transporters (Rees et al. 2009). Each hydrophobic region is followed by a large soluble domain (520 and 370 residues, respectively) located on the cytoplasmic side of the disk membrane. These cytoplasmic regions contain NBDs and are therefore responsible for ATP hydrolysis.

Despite much experimental effort, knowledge of the structural features of ABCA4 is minimal. The main source of full-length ABCA4 for structural and functional studies has been rod outer segments of bovine retina (Ahn and Molday 2000; Ahn et al. 2000; Illing et al. 1997; Sun et al. 1999), where this ~250 kDa protein is present at a molar ratio of about 1:120 to rhodopsin. After isolation of ROS by the well developed sucrose gradient method, ROS membranes are solubilized in detergent and the protein is usually purified to homogeneity in one step by using immunoaffinity chromatography with the Rim3F4 monoclonal antibody (Illing et al. 1997). In a limited number of cases, ABCA4 was also isolated from human post-mortem retinas (Bungert et al. 2001). In addition, many studies have used recombinant human or bovine ABCA4 (89% sequence identity) transiently expressed in mammalian cells, where these proteins localized to the endoplasmic reticulum or intracellular vesicular structures (Ahn et al. 2000, 2003; Sun et al. 2000; Zhong et al. 2009). It is generally assumed that native and recombinant ABCA4 proteins have identical or at least similar properties, but because of limited yields, no thorough studies have been done to assess the folding and structure of ABCA4 obtained from different sources. Moreover, because most studies assume

that this protein is a monomer, possible oligomeric states have not been studied. It has been shown, however, that the closely related ABCA1 transporter forms dimers and may undergo a transition to higher oligomers during the catalytic cycle (Trompier et al. 2006).

The N-terminal and C-terminal moieties of ABCA4 may interact with each other. This was established by comparing the catalytic and nucleotide-binding properties of individually expressed and co-expressed halves of the transporter in mammalian cells (Ahn et al. 2003). It should be noted, however, that the observations made in this study may result in part from protein misfolding.

The results of an early study suggested that ABCA4 may be phosphorylated in a light-dependent manner (Szuts 1985). This possible modification still needs to be explored, especially because it has been shown that phosphorylation of other ABC transporters can regulate transport activity (Noe et al. 2001; See et al. 2002) or mediate degradation (Kolling and Losko 1997; Martinez et al. 2003).

4.4 Structural Features of Individual Domains

Structural features of the exocytoplasmic domains of ABCA4 have not been extensively studied. These domains do not show significant sequence similarity to known proteins, except for other closely related ABCA transporters. An early work showed that bovine and frog ABCA4 are glycosylated and that binding of the Concanavalin A lectin requires disruption of the disk membranes, suggesting that the modified residues are located in the disk lumen (Molday and Molday 1979). In a subsequent study, eight N-linked glycosylation sites were identified in the two exocytoplasmic domains by systematically mutating the predicted sites in human recombinant ABCA4 expressed in COS-1 cells (Fig. 2d) (Bungert et al. 2001). Deglycosylated ABCA4 displayed only a slight decrease in molecular weight as determined by SDS-PAGE, indicating that its sugar chains are small (Azarian and Travis 1997; Bungert et al. 2001; Illing et al. 1997). It was also shown that ECD1 and ECD2 of bovine ABCA4 are linked to each other by at least one disulfide bond, as determined by trypsin digestion under reducing and non-reducing conditions (Bungert et al. 2001). The biological roles of the exocytoplasmic domains of ABCA4 are unknown. In closely related ABCA1 the corresponding "largest extracellular loops" seem to be responsible for interacting with apolipoprotein A-I (Fitzgerald et al. 2002). It is therefore possible that the exocytoplasmic domains of ABCA4 may be involved in interactions with other proteins.

The two cytoplasmic domains of human ABCA4 have been successfully expressed in *E. coli* and isolated from the soluble fraction of cell lysates as well as refolded from insoluble inclusion bodies (Biswas 2001; Biswas and Biswas 2000; Biswas-Fiss 2006; Suarez et al. 2002). Fluorescence anisotropy measurements suggest that these two domains interact in a nucleotide-dependent manner with dissociation constants in the sub-nanomolar range (Biswas-Fiss 2006). A conventional ATP-binding cassette (or NBD) of about 200 residues in size constitutes a part of

each cytoplasmic domain. The structural and functional properties of the remaining ~320 (cytoplasmic domain 1) and ~170 (cytoplasmic domain 2) residues are unclear, although in some ATP transporters these regions carry out regulatory functions (Gerber et al. 2008; Kashiwagi et al. 1995). Mutagenesis studies have demonstrated that a conserved 'VFVNFA' motif located at the C-terminus of cytoplasmic domain 2 is essential for correct folding of ABCA4 (Zhong et al. 2009).

NBDs are the only regions of ABC transporters that are structurally highly conserved. Thus, some insights into the structure of NBDs of ABCA4 can be gained through homology modeling (Cideciyan et al. 2009; Molday et al. 2009) (Fig. 3). Similar to the NBDs of many other ABC transporters, these domains are organized in two distinct halves, a RecA-like subdomain, which is universal for many ATPases, and a smaller helical domain, unique for ABC transporters (Davidson and Chen 2004). The RecA subdomain houses the Walker A and Walker B motifs that participate in binding and coordination of the nucleotide and magnesium atom. Two conserved single-residue motifs, the H-loop and the A-loop, are also important for correct binding of the substrate. Another one-residue motif, the Q-loop, is located

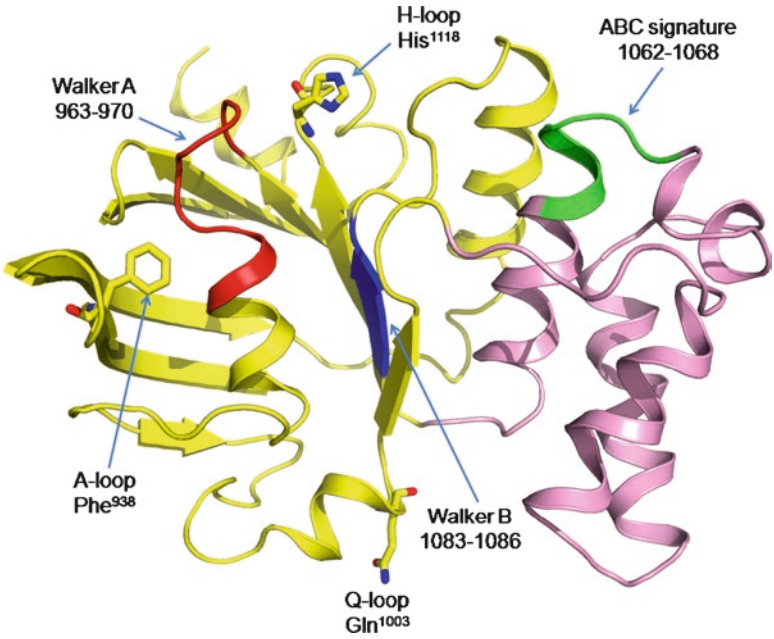

Fig. 3 Cartoon representation of a homology model of NBD1 obtained using SWISS-MODEL server (Kiefer et al. 2009) and refined by energy minimization with the NAMD Molecular Dynamics Simulator (Phillips et al. 2005). The crystal structure of the ABC transporter ATP-binding protein from *Thermotoga maritima* (Protein data bank ID 1VPL, 33.3% sequence identity) was used as a template (Cideciyan et al. 2009). The RecA-like subdomain is *yellow*; the helical domain is *pink*. Walker A, Walker B and ABC signature motifs are *red*, *blue* and *green*, respectively. Single-residue motifs are shown as sticks. The figure was prepared with Pymol (http://pymol.sourceforge.net/)

at the border of the RecA-like and helical domains. In addition to contacting the γ-phosphate of ATP, this loop presumably couples the energy-producing NBDs to the ligand-binding TMDs. It has been firmly established that dimerization of NBDs is essential for substrate translocation by ABC transporters (Davidson and Chen 2004; Kos and Ford 2009; Linton and Higgins 2007; Rees et al. 2009). When in the dimeric state, the ABC signature motif, situated in the helical subdomain of each NBD, participates in formation of the ATP-binding site along with the Walker A motif of the partner NBD.

5 Biological Role of ABCA4

5.1 Identification of Substrate: Biochemical Evidence

Several biochemical studies have been performed to determine the natural substrate of ABCA4. It should be noted, however, that an assay that directly measures active transport across a membrane, as well as reveals the direction of that transport, has yet to be developed. Without such an assay, the reported biochemical evidence is based on the observation that binding of the substrate stimulates the ATPase activity of many ABC transporters. A seminal work measuring the ATP hydrolysis rate of bovine ABCA4 reconstituted in liposomes in the presence of 43 different compounds revealed that isomers of retinal had a two- to fivefold stimulatory effect, whereas all-*trans*-retinol and all-*trans*-retinoic acid showed lower activation efficiencies (Sun et al. 1999). Among other tested substances, amiodarone, digitonin, dehydroabietylamine, and 2-*tert*-butylanthroquinone demonstrated the same level of activation as retinal. Further kinetic analyses, however, suggested that retinal is a transported substrate, whereas the other stimulatory compounds may act as allosteric effectors (Sun et al. 1999). Subsequent studies have focused exclusively on the stimulatory effect of retinal-related compounds. In particular, it was shown that a naturally occurring reversible conjugate of all-*trans*-retinal and phosphatidylethanolamine, *N*-retinylidene-PE, increases the rate of ATP hydrolysis threefold (Ahn et al. 2000). Furthermore, a solid phase assay revealed that the ratio between the bound *N*-retinylidene-PE and ABCA4 is approximately 1:1 and, most importantly, that ATP could release *N*-retinylidene-PE and all-*trans*-retinal from the protein, while ADP and AMP-PNP were far less effective in this regard (Beharry et al. 2004). Based on these studies, it has been accepted that biochemical evidence points to retinal and *N*-retinylidene-PE as transported substrates of ABCA4 (Molday 2007; Molday et al. 2009; Sullivan 2009; Vasiliou et al. 2009). Of note, both the basal and the stimulated ATPase activities of ABCA4 varied significantly from study to study and among protein preparations, displaying a strong dependence on detergent type, the presence and composition of lipids and the presence of a reducing agent among other factors (Ahn et al. 2000; Sun et al. 1999).

The nucleotide specificity of ABCA4 and the roles of the two NBDs have been matters of debate. It was shown that purified native ABCA4 bound ATP and GTP

with similar affinities (Illing et al. 1997) and that the detergent-solubilized full-length protein had ATPase and GTPase activities of about 200 nmol/min/mg (Ahn et al. 2000). Based on functional studies of individually expressed N- and C-terminal halves of the protein, it was also suggested that only NBD2 possesses the nucleotidase activity, while NBD1 has a tightly bound ADP molecule in the active site and does not participate in transport (Ahn et al. 2003). In contrast, the results of a mutagenesis study of full-length ABCA4 expressed in mammalian cells argue that both NBDs are active, but have distinct functions: NBD1 is responsible for basal ATPase activity, whereas NBD2 produces the retinal-stimulated increase in activity (Sun et al. 2000). Finally, a series of papers devoted to analyzing the individual cytoplasmic domains expressed in *E. coli* demonstrated that NBD2 is strictly specific for ATP (Biswas and Biswas 2000), and NBD1 is a general ribonucleotidase that prefers CTP as a substrate (Biswas 2001). It was also suggested that NBD2 may have an inhibitory effect on NBD1 within full-size ABCA4 (Biswas-Fiss 2006).

5.2 Proposed General Model of Transport

Several working models of transport have been suggested for ABCA4 that mostly differ with respect to the roles of NBDs, as described above (Molday 2007; Molday et al. 2009; Sullivan 2009; Sun et al. 2000). The proposed mechanism of transport is based on the 'alternating access' model, established for smaller ABC transporters (Kos and Ford 2009; Linton and Higgins 2007; Rees et al. 2009).

Accumulated biochemical evidence suggests all-*trans*-retinal and *N*-retinylidene -PE as the substrates but provides no clues about the direction of transport. The assumption that ABCA4 translocates the substrate from the luminal to the cytoplasmic side of the ROS disk is thus based on the well established logistics of the visual cycle in rods (Fig. 4), according to which all-*trans*-retinal undergoes reduction to all-*trans*-retinol by all-*trans*-retinol dehydrogenase (atRDH) residing on the cytoplasmic side, and is then transported to the cells of the retinal pigment epithelium (RPE) for further processing. Therefore, ABCA4 is currently believed to be an importer, which makes this protein unique among known eukaryotic ABC transporters. The 'alternating access' model suggests that the TMDs can form two different binding sites for the substrate (Fig. 5). In importers, the high-affinity site is located across the membrane from NBDs. Binding of the substrate to this site supposedly increases the affinity of NBDs for ATP. Upon binding ATP, NBDs come in close contact to form a dimer with the two nucleotide molecules positioned at its interface. This movement induces a conformational transition in TMDs that leads to the closure of the high-affinity substrate binding site and to the translocation of the substrate molecule to the low-affinity site located on the cytoplasmic side of the membrane. Hydrolysis of ATP then separates the NBDs and promotes dissociation of the ADP molecules, thus completing the transport cycle. In the absence of substrate, the transporter undergoes cycles of slow ATP hydrolysis by the individual NBDs, resulting in the basal ATPase activity.

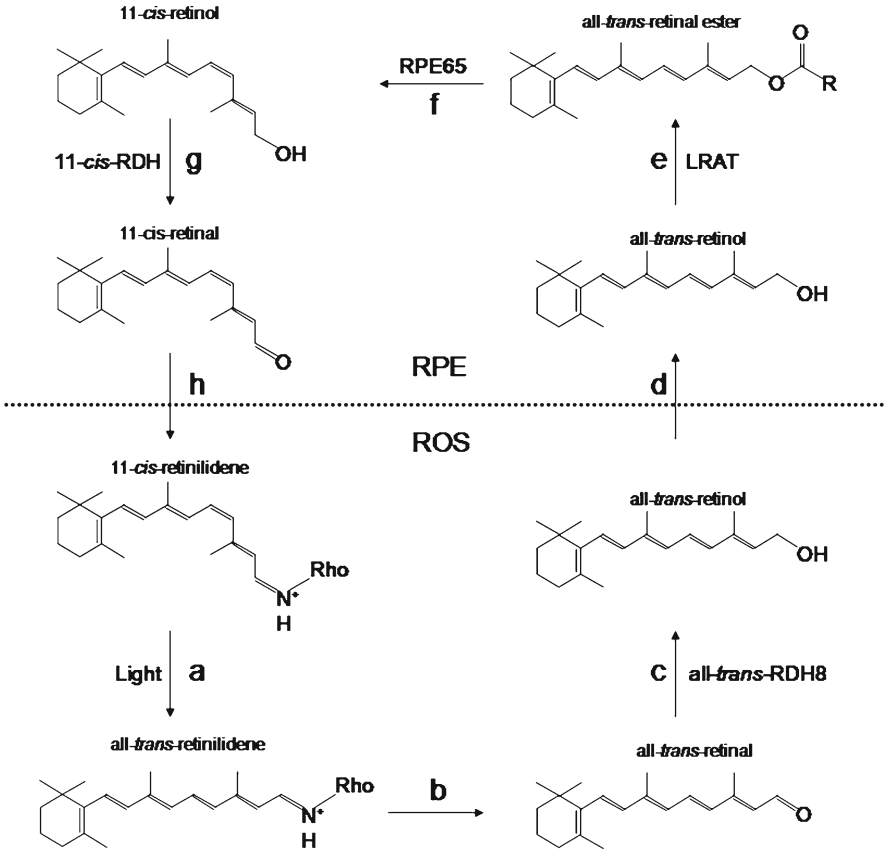

Fig. 4 Transformations of the chromophore in the visual cycle. Light absorption isomerizes 11-*cis*-retinal to all-*trans*-retinal (**a**), followed by dissociation of all-*trans*-retinal from rhodopsin (**b**). All-*trans*-retinal is converted to all-*trans*-retinol by all-*trans*-retinol dehydrogenase 8 located on the cytoplasmic side of the disk membrane (**c**). All-*trans*-retinol is then transported to the RPE (**d**) and esterified by lecithin:retinol acyl transferase (**e**). Rpe65 isomerase converts all-*trans*-retinal ester to 11-*cis*-retinol (**f**). 11-*cis*-Retinol dehydrogenase oxidizes 11-*cis*-retinol to 11-*cis*-retinal (**g**). Finally, 11-*cis*-Retinal is transported back to the ROS where it reassociates with opsin (**h**)

5.3 Abca4 Knockout Mice

Abca4$^{-/-}$ mice (Weng et al. 1999) do not fully reproduce the phenotypes of Stargardt disease and age-related macular degeneration, and some of the described phenotypes were highly exaggerated. These animals exhibit healthy photoreceptors that, under ordinary lighting conditions, show no degradation during their life time. A mild retinal degeneration, however, was induced by exposure to 10,000 lux fluorescent light for 1 h (Maeda et al. 2008), indicating that *Abca4*$^{-/-}$ animals may be more vulnerable to light damage under more extreme conditions. The initial study

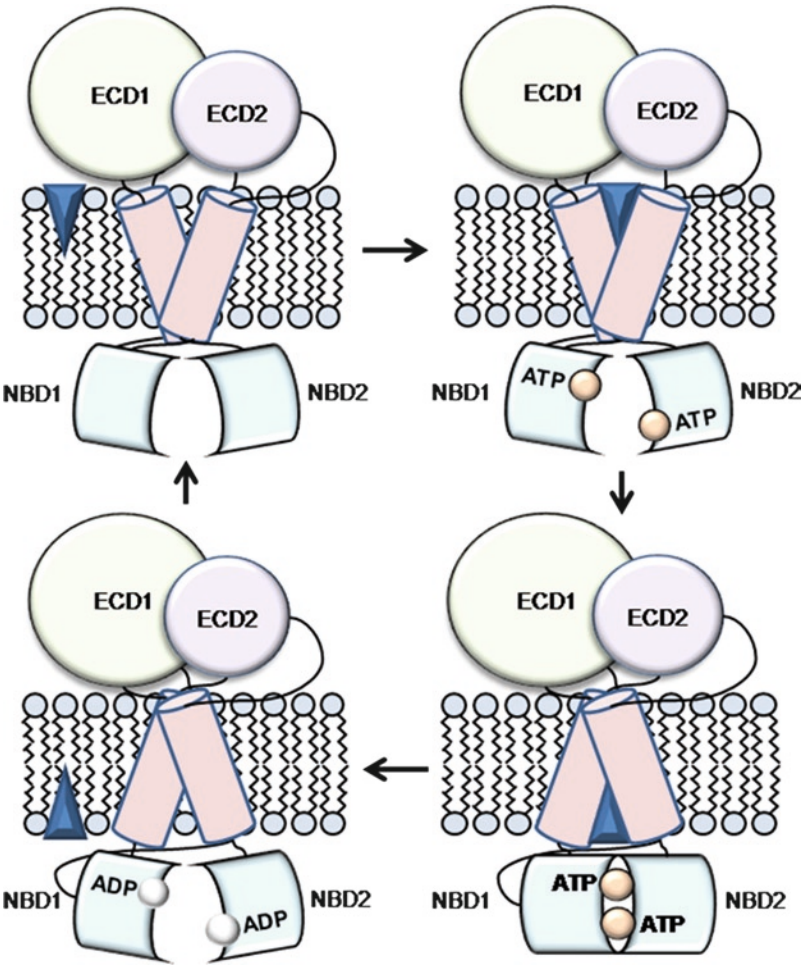

Fig. 5 General model of the ABCA4 transport cycle. The substrate is shown as a *blue diamond*. See text for details

also suggested that delayed dark adaptation, another symptom of Stargardt disease, was present in *Abca4*$^{-/-}$ mice, and that it was initiated by delayed clearance of all-*trans*-retinal, which is known to re-associate with opsin and trigger the phototransduction cascade (Weng et al. 1999). In contrast, others have demonstrated that these animals adapt to the dark even faster than the wild-type mice (Pawar et al. 2008). Likewise, the rates all-*trans*-retinal clearance in *Abca4*$^{-/-}$ and wild-type mice were found comparable by other investigators (Maeda et al. 2008). Hence, the Abca4 knockout mice do not represent a precise model of the human macular degeneration. Notably, a phenotypic response very similar to human AMD and Stargardt disease has been recently observed in double-knockout mice lacking both Abca4 and all-*trans*-retinol dehydrogenase 8 (Rdh8) (Maeda et al. 2009a–c).

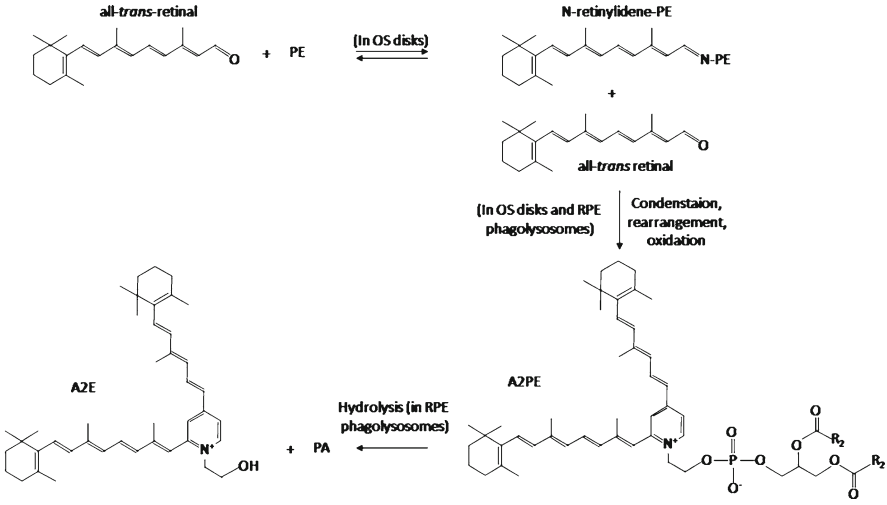

Fig. 6 Formation of A2E, a potentially harmful side product of the visual cycle. In disks of photoreceptor outer segments, all-*trans*-retinal can spontaneously and reversibly react with PE to form *N*-retinylidene-PE. *N*-Retinylidene-PE can condense with a second molecule of all-*trans*-retinal, which, after rearrangement and oxidation, leads to formation of di-retinoid-pyridinium-phosphatidylethanolamine (A2PE). Under acidic conditions in phagolysosomes, A2PE is hydrolyzed to yield di-retinoid-pyridinium-ethanolamine (A2E) and phosphatidic acid (PA)

Despite the lack of retinal degeneration in Abca4 deficient mice, biochemical analyses of retinoid compounds and lipids from ocular tissues revealed alterations consistent with the proposed role of ABCA4 as the transporter of all-*trans*-retinal and/or *N*-retinylidene-PE. Thus, these animals exhibited elevated levels of PE and *N*-retinylidene-PE in the retina (Mata et al. 2000; Weng et al. 1999). Moreover, A2E, the ultimate product of condensation of all-*trans*-retinal and *N*-retinylidene-PE (Fig. 6), and its potentially toxic photoreactive products accumulated in the cells of RPE, accompanied by formation of lipofuscin pigment granules (Mata et al. 2000, 2001; Radu et al. 2004). Importantly, accumulation of A2E and its precursors was found to be strongly light dependent, as *Abca4*$^{-/-}$ mice raised in total darkness did not exhibit these compounds (Mata et al. 2000).

5.4 Proposed Role of ABCA4 in the Visual Cycle

The results obtained from biochemical studies, characterization of *Abca4*$^{-/-}$ mice, and analyses of patients with Stargardt disease have allowed the creation of a conceptual scheme delineating the possible role of ABCA4 in rod cells (Fig. 7a) (Molday 2007; Molday et al. 2009). Absorption of light by rhodopsin leads to isomerization of 11-*cis*-retinal to all-*trans*-retinal, which is then released into the disk membrane. The following step in the visual cycle is reduction of all-*trans*-retinal

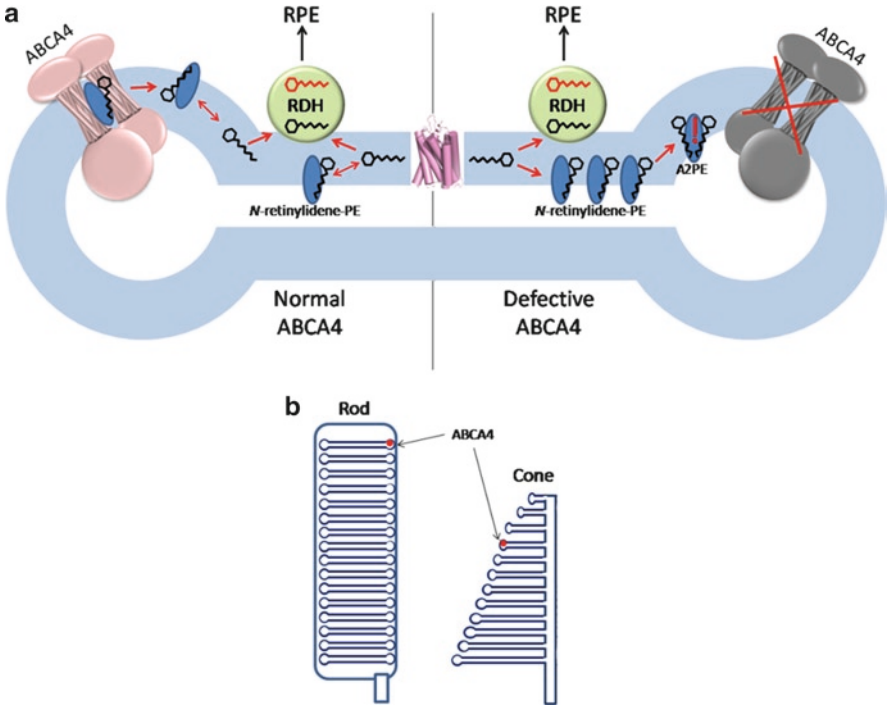

Fig. 7 (**a**) Illustration of the proposed biological role of ABCA4. The *left part* of the diagram represents a ROS disk with a functional ABCA4, whereas the *right part* represents a ROS disk with an inactive ABCA4. Hexagons with carbon backbone denote all-*trans*-retinal (*black*) and all-*trans*-retinol (*red*). RDH: all-*trans*-retinol dehydrogenase 8. RPE: retinal pigment epithelium. See text for the description of the mechanism. (**b**) Schematic representations of rod and cone outer segments. Cone disks are open structures

to all-*trans*-retinol by all-*trans*-retinol dehydrogenase. All-*trans*-retinol dehydrogenase 8 (RDH8), which is located outside of the disk, is responsible for the majority of this activity (Maeda et al. 2007). It has been suggested that ABCA4 may accelerate the clearance of all-*trans*-retinal by translocating it from the luminal to the cytoplasmic side of the disk membrane, but it is not clear if such a hydrophobic substance needs assistance in crossing this lipid bilayer, since it has been shown that retinoids undergo rapid spontaneous transfer between liposomes as well as ROS (Ho et al. 1989; Rando and Bangerter 1982). In addition, ABCA4-mediated transport is relatively slow, with the highest published V_{max} value for ATP hydrolysis being 673 nmol/min/mg (Ahn et al. 2000), equivalent to ~3 enzymatic cycles per second. This may be inadequate for efficient all-*trans*-retinal clearance after strong photobleaching. Moreover, given its hydrophobicity and lack of electric charge, all-*trans*-retinal will probably tend to return to the central part of the membrane unless it is directly passed to RDH8. A much more attractive substrate for ABCA4 is *N*-retinylidene-PE (Fig. 6). This reversible adduct of all-*trans*-retinal and PE

forms spontaneously and cannot cross the membrane by itself. It has been shown that after a 45% photobleach, about 24% of all-*trans*-retinal is present in the form of *N*-retinylidene-PE in wild-type mouse retinas (Mata et al. 2000), whereas in dark-adapted wild-type retinas this fraction reaches nearly 100% (Weng et al. 1999). Hence, the role of ABCA4 could be to flip *N*-retinylidene-PE to the cytoplasmic side of the disk membrane, where it can dissociate and allow all-*trans*-retinal to reenter the visual cycle. By preventing accumulation of *N*-retinylidene-PE inside the disk, ABCA4 also would reduce the reaction of *N*-retinylidene-PE with the second molecule of all-*trans*-retinal, leading to formation of di-retinoid-pyridinium-phosphatidylethanolamine (A2PE) (Eldred and Lasky 1993; Mata et al. 2000). Aged ROS disks are continuously shed and undergo phagocytosis and degradation in phagolysosomes of adjacent RPE cells. In acidic phagolysosomes, A2PE is hydrolyzed to yield A2E (Fig. 6), a major component of lipofuscin that cannot be metabolized further. Because of this, patients with impaired ABCA4 activity progressively accumulate large quantities of A2E in the RPE. This accumulation proceeds faster in the macular region of the retina because of high concentrations of cones in the fovea and rods in the parafovea belt. A2E can have several negative effects on RPE cells, including generation of reactive oxygen species (Jang et al. 2005; Radu et al. 2004), impairment of lysosomal degradative functions (Holz et al. 1999), and by acting at high concentrations as a cationic detergent to perturb biological membranes (Eldred and Lasky 1993). Death of RPE cells leads to the loss of photoreceptors and, therefore, decreased central vision.

5.5 Unresolved Issues

The above described scheme constitutes the best model for the function of ABCA4 available to date. However, several unresolved issues indicate that the biological role of this protein may be more complex and that the current transport model may lack important steps. In particular, it is known that *N*-retinylidene-PE is unstable and exists in equilibrium with all-*trans*-retinal and PE (Ahn et al. 2000). The suggested scheme assumes that RDH8, the main all-*trans*-retinol dehydrogenase of the photoreceptor outer segment, quickly reduces all-*trans*-retinal to all-*trans*-retinol on the cytoplasmic side of the disk membrane, thereby shifting the equilibrium towards dissociation of *N*-retinylidene-PE. However, the RDH8-mediated reduction of all-*trans*-retinal is unexpectedly slow, and likely to be effective in clearing all-*trans*-retinal only at high illumination intensities (Palczewski et al. 1994). If this observation is taken into account, then considerable amounts of A2PE may well form on the outer side of disks.

Another concern arises from important morphological differences between rods and cones (Fig. 7b). Unlike closed ROS disks surrounded by a plasma membrane, cone disks are open structures with contiguous intradiscal and extracellular spaces (Mustafi et al. 2009). It has been shown by electron microscopy that ABCA4 (then known as “a large integral membrane protein”) is present in the margins of cone

disks (Papermaster et al. 1982). Given that NBDs of an ABC transporter must be located inside the cell and assuming that the function of ABCA4 is the same in all types of photoreceptors, it follows that in cones it should transport *N*-retinylidene-PE from outside of the cell into the cytoplasm. The visual cycle in cones is poorly understood, and the rationale for such a translocation needs to be clarified. One explanation is that in cones *N*-retinylidene-PE forms on the extracellular side of the membrane and needs to be transported inside the cell for detoxification.

As of now, the function of ABCA4 in the brain is unknown. It has been speculated that it may have an impact on retinoid modulation of central nervous system function (Kim et al. 2008), but it should be noted that, except for in the eye, retinoids are usually present in tissues as retinyl esters, retinol and retinoic acid rather than as retinal. However, retinol and retinoic acid were found to be poor substrates for ABCA4 (Sun et al. 1999).

6 ABCA4 Mutations and Autosomal Recessive Macular Degeneration

Disease-associated alleles of Abca4 are unusually heterogeneous, with about 400 mutations described so far, of which most represent missense substitutions (Allikmets 2000; Lewis et al. 1999). In addition, the most frequent alleles are found in less than 20% of patients, making the search for correlations between individual mutations and disease severity more difficult. Moreover, establishing a reliable method for estimating disease severity represents an additional challenge (Cideciyan et al. 2009).

Mutations are evenly distributed throughout the primary structure of ABCA4. Several studies focused on investigating the effect of amino acid substitutions and deletions on activity of the full-length protein (Sun et al. 2000; Zhong et al. 2009) and its individual cytoplasmic domains (Biswas and Biswas 2000; Biswas-Fiss 2003, 2006; Suarez et al. 2002). Some of these substitutions were found to have a pronounced effect on the ATPase activity of the NBDs (Biswas and Biswas 2000; Biswas-Fiss 2003, 2006; Suarez et al. 2002; Sun et al. 2000), whereas others resulted in reduced expression levels (Sun et al. 2000; Zhong et al. 2009). Establishing the effects of many mutations is currently impossible because of the lack of a suitable transport assay, since only those mutations that alter ATPase activity can be described.

In light of ABCA4 involvement in several visual disorders, a model was proposed in which the severity of the disease in any given individual is inversely correlated with the residual activity of the mutant ABCA4 proteins (Shroyer et al. 1999; van Driel et al. 1998). Recent studies have shown that the residual function hypothesis is oversimplified. In particular, a study in transgenic frogs revealed that some of the mutants retained ABCA4 in the inner segments of their photoreceptors, indicating that protein mislocalization can contribute to the severity of the disease (Wiszniewski et al. 2005). Furthermore, it was recently demonstrated by statistical

analysis of data collected on a large cohort of patients over a long period of time that several individuals with two missense or splicing mutations developed much more severe phenotypes than those with two truncating mutations (Cideciyan et al. 2009). This result clearly contradicts the residual function model, according to which truncations represent the most detrimental mutations because they prevent protein expression. Therefore, models involving genotype-phenotype correlations should account for the negative effects of ABCA4 misfolding and mislocalization along with reduced activity.

7 Conclusions

ABCA4 is a member of the superfamily of ATP-binding cassette transporters expressed primarily in vertebrate photoreceptors, where it localizes to the rims of outer membrane disks (rods) and evaginations (cones). A combined effort including biochemical, clinical and animal model studies has highlighted its role in clearance of all-*trans*-retinal from the disk membranes after photoexcitation of rhodopsin. The most probable substrate of ABCA4 is *N*-retinylidene-PE, a product of the reaction of all-*trans*-retinal with phosphatidylethanolamine. If not removed from disk membranes, *N*-retinylidene-PE can further react with a second molecule of all-*trans*-retinal to form potentially harmful diretinal compounds. During the process of disk shedding, these compounds, of which the best studied is A2E, accumulate in the cells of RPE, which ultimately leads to RPE cell death and concomitant degeneration of photoreceptors.

Despite impressive progress that has been achieved in understanding the function of ABCA4 in vision, a number of important problems remain. Creation of a transport assay is critical for verification of the proposed substrates and determination of the direction of transport for ABCA4. The role of ABCA4 in brain awaits resolution. Finally, biochemical and structural studies should be undertaken to gain insights into the mechanism of ABCA4-mediated substrate translocation and its regulation.

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Chapter 9
Suppression of Drusen Formation by Compstatin, a Peptide Inhibitor of Complement C3 activation, on Cynomolgus Monkey with Early-Onset Macular Degeneration

Zai-Long Chi, Tsunehiko Yoshida, John D. Lambris, and Takeshi Iwata

Abstract For the past 10 years, number of evidence has shown that activation of complement cascade has been associated with age-related macular degeneration (AMD). The genome wide association study in American population with dominantly dry-type AMD has revealed strong association with single nucleotide polymorphism (SNP) of complement genes. Protein composition of drusen, a deposit observed in sub-retinal space between Bruch's membrane and retinal pigment epithelial (RPE), contains active complement molecules in human and monkey. These evidences have leaded us to consider the possibility of suppressing complement cascade in the retina to delay or reverse the onset of AMD. To test is hypothesis we used the C3 inhibitor Compstatin on primate model with early-onset macular degeneration which develop drusen in less than 2 years after birth. Our preliminary result showed drusen disappearance after 6 months of intravitreal injection.

1 AMD and Association of Complement Related Genes

The most prevalent eye disease for elderly Europeans and Americans is AMD. AMD is a blinding disorder characterized by a marked decrease in central vision associated with retinal pigment epithelial (RPE) atrophy with or without choroidal neovascularization (CNV). The non-neovascular type is called the dry-type AMD and includes more than 80% of the cases, and the neovascular type is called the wet-type AMD which is progressive with a higher probability of blindness. In some cases of CNV, the new vessels penetrate Bruch's membrane and pass into the sub-retinal space. The progressive impairment of the RPE and damage to Bruch's membrane and choriocapillaris results in retinal atrophy and photoreceptor dysfunction.

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Genetic, behavioral, and environmental factors are believed to be involved for the onset of this disease. The prevalence of AMD differs considerably among the different ethnic groups, but the incidence increases with age in all groups. Epidemiological studies have shown that genetic factor play critical role for AMD. However, only a small proportion of the families with AMD show Mendelian inheritance, and the majority of the individuals inherit AMD in a complex multi-gene pattern. With the help of the haplotype marker project (HapMap Project), genome wide scanning has identified at least 13 loci linked to AMD on different chromosomes (Iyengar et al. 2004; Schick et al. 2003; Majewski et al. 2003). Other risk factors such as cigarette smoking, obesity, hypertension, and atherosclerosis are also associated with the disease.

Recently, a polymorphism of complement factor H (CFH) gene (Y402H) was shown to be associated with an increased risk for AMD (Klein et al. 2005; Edwards et al. 2005; Haines et al. 2005; Hageman et al. 2005). These results were confirmed in many of the countries with large Caucasian populations but not in Japan (Okamoto et al. 2006; Gotoh et al. 2006). This gene is located on chromosome 1q25–31 where one of the candidate loci was identified by whole genome association studies by linkage markers. Another recent study reported that a haplotype association of tandemly located complement 2 and factor B (Gold et al. 2006) was protective and C3 (Yates et al. 2007) as risk for AMD. HTRA1, a serine protease 11 was recently discovered to be strongly associated with AMD (Yang et al. 2006; Dewan et al. 2006). Unlike the CFH, our study shows strongly association with this gene for Japanese AMD patients (Yoshida et al. 2007). This difference of gene association is probably related to the difference of AMD type dominant in each country. Our genome wide association study on Japanese population with typical wet-type AMD and polypoidal choroidal vasculopathy (PCV) shows significant association at p-value of 10^{-14} and 10^{-7} respectively for ARMS2/Htra1 locus. However when much lower associated SNPs of CFH or C3 or combined the odds ratio significantly increased (Goto et al. 2009)

2 Activated Complement Component in Drusen

The early stage of the dry-type AMD is characterized by thickening of Bruch's membrane, aggregation of pigment granules, and increasing numbers of drusen. Drusen are small yellowish-white deposits that are composed of lipids, proteins, glycoproteins, and glycosaminoglycans. They accumulate in the extracellular space and the inner aspects of Bruch's membrane. Drusen are not directly associated with visual loss but represent a risk factor for dry-type AMD. The classification of hard and soft drusen is based on their size, shape, and color; hard drusen are yellowish with diameters <50 μm and are found in eyes that are less likely to progress to advanced stages of the disease, while soft drusen are darker yellow and larger in size, and are found in eyes more likely to progress to more advanced stages of AMD.

Both immunohistochemistry and proteomic techniques have shown that drusen are composed of molecules that mediate inflammatory and immune processes (Russell et al. 2000; Mullins et al. 2000). These molecules include components of the complement pathway and modulators of complement activation, viz., vitronectin, clusterin, membrane cofactor protein, and complement receptor-1. In addition, molecules triggering inflammation, amyloid P component, α1-antitrypsin, and apolipoprotein E, were identified in drusen. Cellular debris from macrophages, RPE cells, and choroidal dendritic cells has been also identified in drusen. Additional proteins such as crystallins, EEFMP1, and amyloid-beta have been found in drusen. The presence of immunoreactive proteins and the oxidative modifications of many proteins in drusen imply that both oxidation and immune functions are involved in the pathogenesis of AMD. Finding of these molecules suggest that complement activation triggers innate immune responses in the subretinal space.

3 Cynomolgus Monkey with Early-Onset Macular Degeneration

Over the past years non-human primates with well-defined fovea has been the target for AMD research. A monkey with macular degeneration was first described by Stafford et al. in 1974. They reported that 6.6% of the elderly monkeys they examined showed pigmentary disorders and drusen-like spots (Stafford et al. 1984). We also observed at approximately the same rate of disorder in elderly cynomolgus monkeys in the Philippines primate facility (SICONBREC) (Umeda et al. 2005a, b). El-Mofty et al. (1978) reported that the incidence of maculopathy was 50% in a colony of rhesus monkeys at the Caribbean Primate Research Center of the University of Puerto Rico. In 1986, a single cynomolgus monkey (*Macaca fascicularis*) with large number of small drusen in the macula was found in Tsukuba Primate Research Center at Tsukuba City, Japan (Nicolas et al. 1996a, b; Suzuki et al. 2003). This single affected monkey has been bred to a large pedigree of more than 300 monkeys (Fig. 1). Drusen are observed in the macula as early as 2 years after birth, and the number increase and spread toward the peripheral retina throughout life (Figs. 2–3). Histological abnormalities of the retina and abnormal electroretinogram (ERG) were observed in sever case showing physiological dysfunction of the macula.

Immunohistochemical and proteomic analyses of the drusen from these monkeys showed that the drusen were very similar to those in other monkeys with aged macular degeneration sporadically found in older monkeys and also with human drusen (Umeda et al. 2005a, b; Ambati et al. 2003). These observations have shown that TPRC monkeys produce drusen that are biochemically similar to those in human AMD patients, but the development of the drusen occurs at an accelerated rate.

More than 240 loci are being investigated to try to identify the disease causing gene and to understand the biological pathways leading to complement activation. Simultaneously, we have been studying a colony of aged monkeys in SICONBREC,

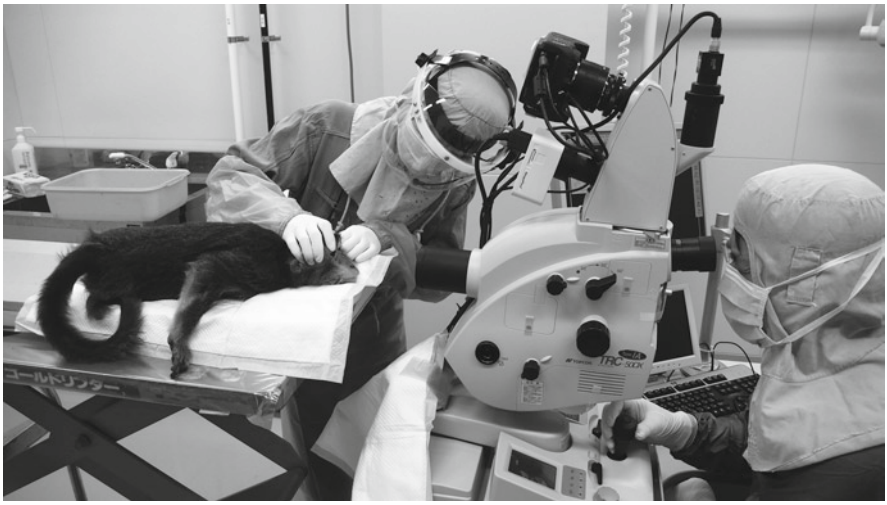

Fig. 1 Fundus photography of affected monkey at TPRC

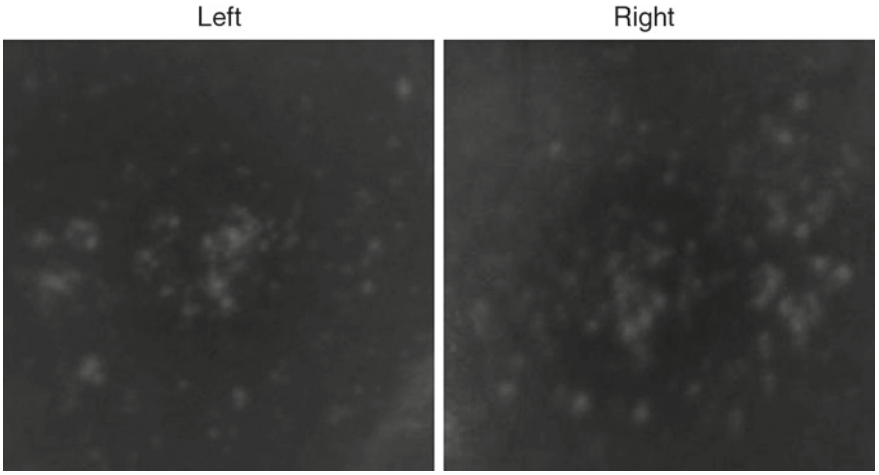

Fig. 2 Fundus photograph of affected monkey showing accumulation of drusen in macula of both eyes

which develop drusen after 15 years of birth. Drusen components of these sporadically found affected monkeys were compared with human and TPRC monkeys by immunohistochemistry and proteomic analysis using ion spray mass spectrometer. Significant finding was that drusen contained protein molecules that mediate inflammatory and immune processes. These include immunoglobulins, components of complement pathway, and modulators for complement activation (e.g., vitronectin, clusterin, membrane cofactor protein, and complement receptor-1), molecules involved in the acute-phase response to inflammation (e.g., amyloid P component,

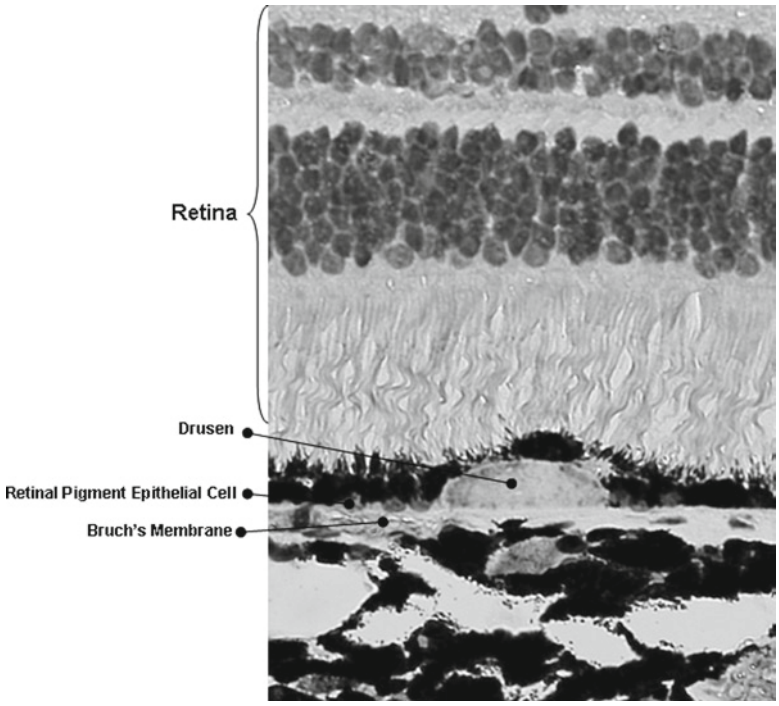

Fig. 3 Retinal histological section of affected monkey showing the accumulation of drusen

α1-antitrypsin, and apolipoprotein E), major histocompatibility complex class II antigens, and HLA-DR antigens (Umeda et al. 2005a, b). Cellular components have also been identified in drusen, including RPE debris, lipofuscin, and melanin, as well as processes of choroidal dendritic cells, which contribute to the inflammatory response. The presence of immunoreactive proteins and oxidative modified proteins implicate both oxidation and immune functions in the pathogenesis of affected monkeys.

4 Suppression and Reversal of Drusen Formation by Compstatin

To test the effect of long term suppression of complement activation in the retina, an cyclic analogue (Ac-I[CV(1MeW)QDWGAHRC]T-NH2) of the small cyclic synthetic peptide compstatin (Katragadda et al. 2006) was intravitreally injected into eight affected monkeys at different dose and intervals. Four affected monkeys were injected at 1 mg dose at 1 month interval while other four affected monkeys at 50 μg dose at 1 week interval. Both 1 mg or 50 μg dose were dissolved in 100 μl of saline solution, filtrated and intravitreally injected using 30G needle.

Due to the unique molecular characteristic of compstatin, immediately after injection, compstatin precipitate and form gel-like structure in the vitreous. This gel will gradually dissolve and disappear after 6 months. Four monkeys injected with 1 mg for 3 months developed significant opacity to the point where fundus observation was impossible. These monkeys were halted for further injection. On the other

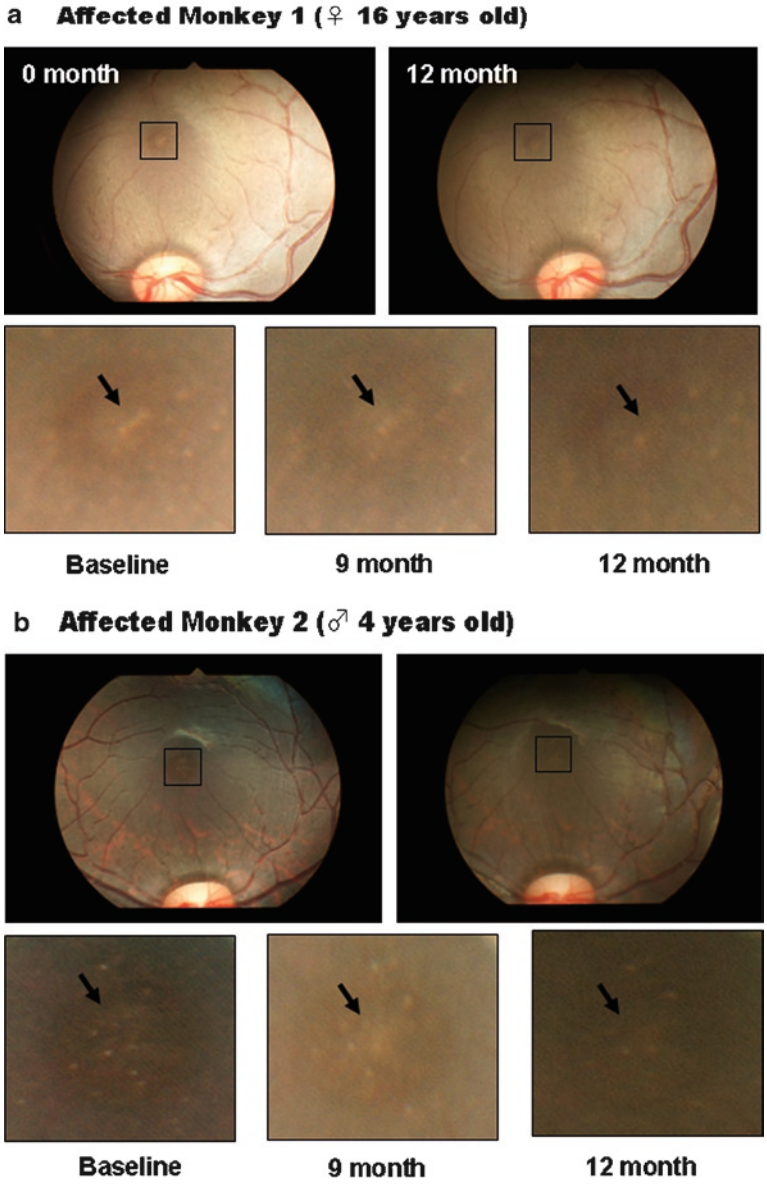

Fig. 4 Suppression and reversal of drusen formation after 9 months of intravitreal injection of 50 μl compstatin at 1 week interval

hand, vitreous of four monkeys with 50 μg dose were clear within 2 days. After 6 months of injection, we noticed diffusion of drusen in the macula and by 9 months partial disappearance of drusen was observed in all four monkeys (Fig. 4). This preliminary experiment has shown reversal of drusen formation by suppression of complement activation. To explain this reversal phenomenon, which has not been observed in untreated affected monkeys, will require further experiments including identification of disease causing gene and pathway leading to complement activation. The information should benefit for development of improved drug and therapy for future AMD prevention.

All experimental procedures for this primate study were approved by the Animal Welfare and Animal Care Committee of the TRPC and the Experimental Animal Committee of the National Tokyo Medical Center. The facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). Monkeys were routinely examined for physical and ophthalmic conditions by veterinarians and by ophthalmologists, respectively.

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Chapter 10
A Targeted Inhibitor of the Complement Alternative Pathway Reduces RPE Injury and Angiogenesis in Models of Age-Related Macular Degeneration

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Abstract Genetic variations in complement factor H (fH), an inhibitor of the complement alternative pathway (CAP), and oxidative stress are associated with age-related macular degeneration (AMD). Recently, novel complement therapeutics have been created with the capacity to be "targeted" to sites of complement activation. One example is our recombinant form of fH, CR2-fH, which consists of the N-terminus of mouse fH that contains the CAP-inhibitory domain, linked to a complement receptor 2 (CR2) targeting fragment that binds complement activation products. CR2-fH was investigated in vivo in the mouse model of choroidal neovascularization (CNV) and in vitro in oxidatively stressed RPE cell monolayers. RPE deterioration and CNV development were found to require CAP activation, and specific CAP inhibition by CR2-fH reduced the loss of RPE integrity and angiogenesis in CNV. In both the in vivo and in vitro paradigm of RPE damage, a model requiring molecular events known to be involved in AMD, complement-dependent VEGF production, was confirmed. These data may open new avenues for AMD treatment strategies.

1 Age-Related Macular Degeneration and Complement Activation

Age-related macular degeneration (AMD) is characterized by progressive loss of central vision resulting from damage to the photoreceptor cells in the central area of the retina, the macula. AMD occurs in two forms: wet and dry; with the dry form making up 80–90% of total cases (Brown et al. 2005). Both forms are associated with pathology

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at the RPE/choroid interface, which leads to subsequent loss of photoreceptors. Structural changes in AMD include a thickening of Bruch's membrane, which is essentially the deposition of extracellular material between the RPE and Bruch's membrane (sub-RPE deposits and drusen), and deterioration of the blood-retina barrier. Dry AMD leads to the slow degeneration and atrophy of the photoreceptors in the macula by mechanisms not fully understood. Wet AMD is associated with choroidal neovascularization (CNV) in the area of the macula and leakage of these new vessels. The accumulation of blood and fluid can cause retinal detachment, followed by rapid photoreceptor loss. Because of the structural differences, wet AMD causes the most severe acute visual loss, while dry AMD is associated with progressive slow visual loss.

AMD is a complex multifactorial disease and may actually represent a family of diseases that affect the macula (see *Retnet.org* for genes associated with autosomal dominant, autosomal recessive or X-linked macular degeneration). The disease process appears to be influenced by a number of environmental insults, making it difficult to identify one central etiology. Indeed, the genetics of macular degeneration and other forms of retinal degeneration point to multiple targets and multiple causes, each of which may be involved in a subset of patients, and ultimately lead to the common endpoint of failed central vision. The primary risk factor for AMD is aging. The only environmental agent unequivocally linked to AMD is nicotine (Schmidt et al. 2006), presumably by generating oxidative stress. Overall, oxidative stress is thought to be an important risk factor, presumably impacting RPE function (Snodderly 1995). Both nicotine and oxidative stress enhance the development of experimental CNV in mice (Suñer et al. 2004; Dong et al. 2009). Finally, complement-activation and the resultant inflammatory response have been proposed as a mediator of all forms of AMD pathology (e.g., Johnson et al. 2001; Klein et al. 2005; Umeda et al. 2005).

Early AMD is characterized by the formation of drusen at the choroid/RPE interface (Johnson et al. 2001; Anderson et al. 2002; Chong et al. 2005; Hageman et al. 2001). Drusen contain a large number of proteins including amyloid proteins, coagulation factors and complement components. A high concentration of complement regulatory proteins, C3 (complement component 3) activation fragments, and membrane attack complex (MAC) proteins, in the area of Bruch's membrane and the RPE, have been documented (Johnson et al. 2001; Hageman et al. 2001; Kijlstra et al. 2005; Lommatzsch et al. 2008). MAC deposition density is correlated with AMD severity and is most pronounced in the macula. Furthermore, there is a correlation between the amount of MAC deposition and the loss of RPE cells (Hageman et al. 2001). Hageman has also demonstrated that local synthesis of complement alternative pathway (CAP) components occurs in AMD, just as others have demonstrated local upregulation of CAP components in other complement-mediated diseases (Girardi et al. 2003). Finally, mRNA and protein expression of fH (complement factor H) and other complement control proteins is increased in AMD eyes. These data, combined with the relative lack of immunoglobulins in drusen (which would activate the classical pathway) (Crabb et al. 2002), support the hypothesis that the CAP plays a critical role in driving the pathology of AMD. Additional support linking CAP to AMD comes from the data that MPGN type II, a disease associated with increased CAP

activity (Mullins et al. 2001; Appel et al. 2005), is associated with the early drusen formation, which is indistinguishable from that in AMD.

The hypothesis that the CAP is critical to AMD pathogenesis was strengthened significantly by reports showing that a polymorphism in the CAP control protein fH is strongly associated with AMD. This polymorphism in fH, a mutation at position 402 from tyrosine to histidine, is associated with a 2 to 2.5-fold increased risk of AMD in heterozygotes and a 5–5.5 increased risk in homozygotes (Klein et al. 2005; Hageman et al. 2005; Haines et al. 2005; Edwards et al. 2005). Analysis suggested that this and other polymorphisms may account for up to 40–60% of the genetic risk for AMD. Since the original report on fH as a risk factor, variations in the genes for fB (complement factor B), C2 (complement component 2), and C3 (Gold et al. 2006; Yates et al. 2007) as major risk factors have also been reported. Thus, the concept emerges that abnormalities in controlling CAP may lead to inflammation, drusen formation and the development of AMD. Here we wished to address two essential questions: (1) do oxidative stress and complement act synergistically in the disease process and generate an environment at the RPE-Bruch's membrane interface that is conducive to AMD pathology; and (2) can we target the complement cascade therapeutically and potentially ameliorate AMD.

2 Targeted Complement Inhibition

Recently, novel complement therapeutics have been created with the capacity to be "targeted" to sites of complement activation (Song et al. 2003). These inhibitors consist of an amino-terminal domain encoding the iC3b/C3d binding-site from complement receptor 2 (CR2/CD21), a region whose structure-function relationships are increasingly well understood, and a carboxy-terminal domain encoding complement inhibitors that are typically membrane-bound, but whose external regulatory domains are linked to CR2. Most of the published data utilizes a recombinant protein consisting of the CR2 domain at the amino terminus followed by the complement regulatory domain of the murine (mu) membrane protein Crry (Song et al. 2003; Atkinson et al. 2005), a protein that demonstrates the ability to block both the classical and alternative pathway at the C3-activation step (Molina et al. 1992). The use of muCR2-Crry, has been reported in several in vivo disease models where it has shown: (1) accumulation of the inhibitor at sites of initial tissue iC3b/C3d deposition, consistent with the effect of the CR2 domain mediating binding to these tissue-bound ligands; (2) protection from complement-dependent tissue injury using 10- to 20-fold less protein than non-targeted complement-based therapeutics such as Crry-Ig; (3) prolonged tissue half-life associated with a relatively short circulating half-life, consistent with binding to local tissues where the CR2 ligands are generated; and (4) no increase in susceptibility to infection, when used at equivalent therapeutic doses as the non-targeted Crry-Ig. Another important effect of the CR2-targeted approach is a decrease in autoantibody generation

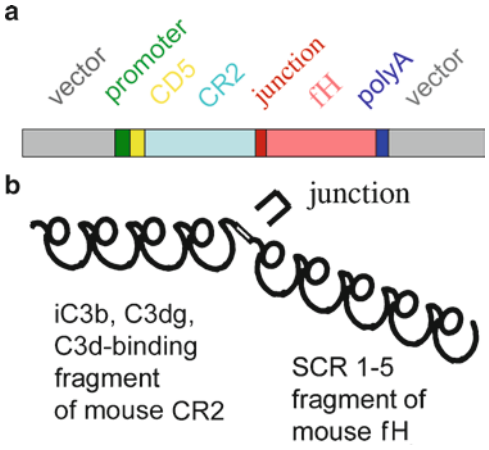

Fig. 1 (**a**) Schematic representation of plasmid encoding CD5 signal peptide, CR2 domain, followed by a linker and 5 extracytoplasmic short consensus repeats of CFH. (**b**) Diagram of CR2-fH fusion protein

that is likely to be due to the inhibition of endogenous B-cell CR2-function by the soluble CR2 portion of the molecule (Song et al. 2007).

In addition to the multi-pathway inhibitor, muCR2-Crry, a CAP-specific CR2-targeted inhibitor has been developed. This CAP inhibitor utilizes the same CR2 domain to allow targeting to sites of initial iC3b/C3d deposition, but links this domain to the amino terminal five SCRs of mouse fH, which encodes the regulatory domain of the molecule (Fig. 1) (Pangburn 2000). CR2-fH has demonstrated therapeutic efficacy in CAP-dependent models of intestinal ischemia-reperfusion injury (Huang et al. 2008). These studies have shown that CR2-fH is able to block C3 deposition on zymosan at least 20-fold more effectively, than endogenous fH or recombinant fH SCRs1-5 without the CR2 domain (Huang et al. 2008). The necessity for the CR2 portion of this chimeric molecule has been confirmed by the reversal of the effect of CR2-fH on hemolysis and zymosan-mediated CAP activation using anti-CR2 mAb 7G6, which blocks the interaction of CR2 with iC3b/C3d (Huang et al. 2008).

3 Oxidative Stress Renders RPE Susceptible to Complement Attack

To examine the effects of complement activation on RPE cells, we turned to an in vitro model, using RPE monolayers (ARPE-19 cells). RPE cells were plated on permeable membrane inserts in the presence of 10% serum; after the cells attached, serum was reduced to 1%. Under these conditions, RPE cells become polarized, form tight monolayers, and stain for markers of tight and adherence junctions

(Bailey et al. 2004; data not shown). These monolayers exhibit stable transepithelial resistance (TER), which can be monitored with an epithelial volt-ohm-meter equipped with an STX2 electrode. Absolute TER values are obtained within 2–3 weeks after reaching confluency (40–45 $\Omega\,cm^2$).

Oxidative stress is one of the environmental risk factors for AMD (Snodderly 1995); however, it is unclear how long-term oxidative stress is involved in generating AMD pathology. Here we asked two related questions; first, whether oxidative stress and complement act synergistically in the disease process; and second, whether oxidative stress facilitates complement activation. Two readouts were analyzed here, TER and VEGF secretion.

Mild oxidative stress was used to treat the cells. We define mild oxidative stress as an amount of stress that does not alter TER by itself. Bailey and colleagues (Bailey et al. 2004) have shown that ARPE-19 cells, when grown in monolayers with stable resistance, can withstand H_2O_2 treatment up to a concentration of 1 mM. Hence, all the experiments were performed with 0.5 mM H_2O_2 applied to the apical side (retina side). TER was found to be resistant to 0.5 mM H_2O_2, whereas the treatment with 10 ng of VEGF/mL resulted in a rapid loss of TER (Ablonczy and Crosson 2007) (positive control; data not shown). TER, however, dropped quickly by 50 ± 2% in response to 0.5 mM H_2O_2 in the presence of 25% complement-sufficient serum. Heat-inactivated, complement-inactivated serum had no effect on TER. This effect required C7 (complement component 7), an essential component of the MAC, as C7-depleted serum + H_2O_2 did not result in TER deterioration (Fig. 2b). Reduction in TER was paralleled by VEGF secretion (Fig. 2a). Importantly, we have confirmed using TUNEL, that RPE cells do not undergo cell death; in addition, no necrotic cells were observed in the bright-field images. These results fulfill the basic criteria of complement-derived sublytic signaling activity, i.e., activation of the complement cascade and the requirement of MAC formation, but no measurable cell death.

Local complement activation is likely a result of the fine balance between the levels of the complement activation pathway proteins (the "fuel"), and the competing effects of activating and inhibitory molecules. To measure complement activation, flow cytometry was used to assess fixation of C3 to ARPE-19 cells after serum challenge. Comparing cells with or without H_2O_2-pretreatment, revealed that the treatment significantly increased C3 binding by ~2.5-fold (262 ± 62% of that seen with untreated cells; $P < 0.05$, $n = 3$) (Thurman et al. 2009). To determine whether increased C3 binding is correlated with reduced levels of endogenous complement inhibitors, antibodies against CD55, CD59 and CD46 were used. Interestingly, oxidative stress reduces the level of both CD55 (Fig. 3) and CD59 (Fig. 3), but not CD46 on the surface of RPE cells. Surface levels of CD55 decreased after treatment with H_2O_2 to ~70% of those seen on unmanipulated cells ($P < 0.001$); mean CD59 expression fell to ~60% (Fig. 3; $P < 0.001$) (Thurman et al. 2009). These data suggest that oxidative stress results in a reduction of endogenous complement inhibitors, allowing this increase in C3 deposition.

If the reduction in endogenous complement inhibition due to the decreased levels of CD55 and CD59 were to be responsible for reduction in TER and VEGF secretion, adding an exogenous complement inhibitor should blunt the effect of H_2O_2 + serum.

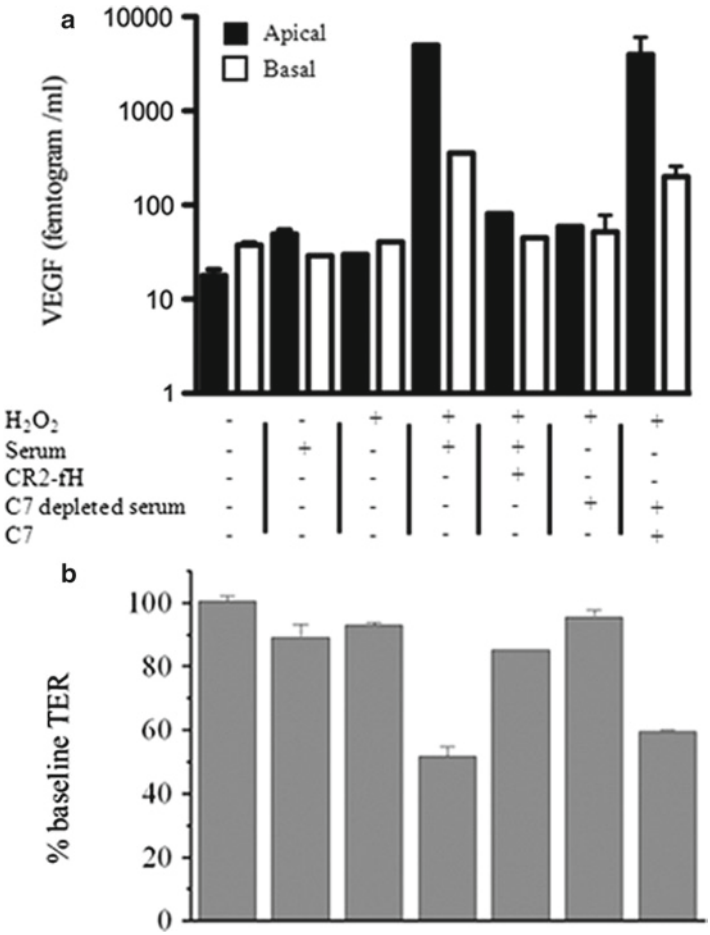

Fig. 2 Complement activation of ARPE-19 cells induces the secretion of VEGF and disruption of barrier function. ARPE-19 cells were grown as a monolayer until a stable TER was obtained. Experimental conditions are indicated by the table, which applies to both halves of the figure. Data is expressed as mean ± stdev. ($n = 3$–6 per condition). (**a**) Following 4 h of apical treatment, both apical and basal supernatants were removed and analyzed for VEGF content by ELISA. At baseline, VEGF secretion is polarized, with increased secretion into the basal compartment. H_2O_2 and serum alone had no consistent effect on VEGF secretion, whereas H_2O_2 + serum increased VEGF release by ~100-fold towards the apical and ~50-fold towards the basal side. H_2O_2 + serum-mediated VEGF release can be inhibited by blocking the CAP (CR2-fH). VEGF secretion was eliminated when C7-depleted serum was applied to oxidatively stressed cells, but was triggered when C7-depleted serum was reconstituted with purified C7, demonstrating that MAC formation is involved in VEGF secretion by these cells. (**b**) Although treatment with H_2O_2 or serum alone did not cause a significant drop in the TER, combined treatment with H_2O_2 and serum did cause the TER to deteriorate. Co-administration of CR2-fH prevented the decline in TER, as did elimination of C7 (data from Thurman et al. 2009)

To test this hypothesis, we pretreated RPE monolayers exposed to H_2O_2 + serum with CR2-fH, which significantly blunted both the reduction in TER (Fig. 2b) and the increased secretion of VEGF (Fig. 2a).

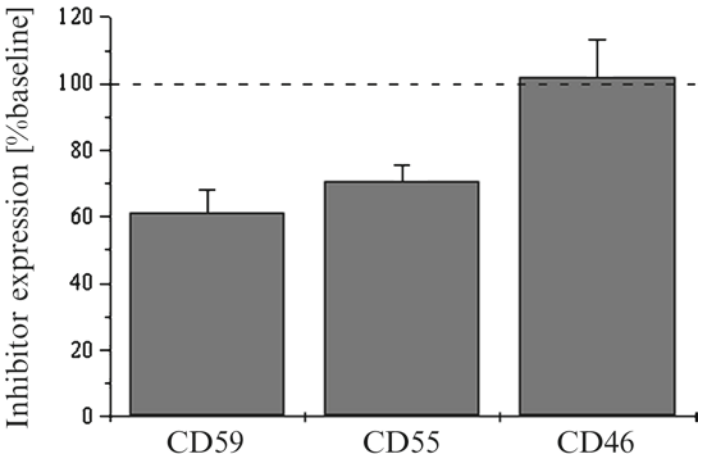

Fig. 3 Oxidative stress reduces the surface expression of DAF and CD59. Surface expression of DAF, MCP, and CD59 was demonstrated on ARPE-19 cells by flow cytometry, using isotype antibodies as control. Surface expression of DAF and CD59 decreased after treatment of the cells for 1 h with 1 mM H_2O_2, whereas surface expression of MCP did not change (data from Thurman et al. 2009)

4 Choroidal Neovascularization is Amenable to CR2-fH Therapy

Finding or developing a therapeutically predictive model for a human disease is critical for the development of successful therapies. The most comprehensively studied animal model for wet AMD is the CNV model in rodents. In this model, injury is induced by argon laser photocoagulation, which ruptures Bruch's membrane and causes loss of RPE and underlying photoreceptors (Nozaki et al. 2006). This injury triggers the subsequent development of vascular proliferation and CNV, which can be visualized by using imaging techniques (Nozaki et al. 2006; Espinosa-Heidmann et al. 2002; Campa et al. 2008). Loss of photoreceptors is documented in retinal sections or using electroretinography. The involvement of the complement system in mouse CNV has been well-documented, with an especially important role for the CAP in inducing C3a, C5a and MAC-dependent increases in CNV (Nozaki et al. 2006; Bora et al. 2006; Kleinman et al. 2008; Bora et al. 2007). Whatever the mechanism of induced injury, however, all studies agree that VEGF is an essential downstream molecule required for the development of CNV. In addition, relevant to our hypothesis, mouse CNV has been shown to be promoted by oxidative stress (Dong et al. 2009).

To test whether an exogenous administered CAP inhibitor could reduce CNV, CR2-fH (250 μg per injection) or the control (PBS or equimolar CR2) were administered via tail vein injections at the time of laser damage (day 0), followed by two additional treatments on days 2 and 4. Mice deficient in alternative pathway activation ($fB^{-/-}$ mice) were used as a positive control. CNV was reduced by ~65% in

fB$^{-/-}$ when compared to wild type mice (Fig. 4a). This value was considered the maximum complement-dependent effect. Treatment with CR2-fH significantly reduced the size of CNV by 40% based on isolectin B staining (CR2-fH vs. PBS or CR2, $P=0.002$; Fig. 4a), and there was no difference in the CNV size when comparing PBS with CR2 ($P=0.3$; Fig. 4a) (Rohrer et al. 2009). CR2-fH treatment significantly preserved retinal function as demonstrated by ERG analysis when compared to PBS. The four lesions caused a reduction in ERG amplitudes to ~60% of the baseline values in wild type animals; eliminating complement activation either genetically (*fB*$^{-/-}$ mice) or pharmacologically (CR2-fH treatment) reduced ERG amplitudes only to ~80% ($P<0.01$) (Rohrer et al. 2009). Other readouts such as mRNA expression for C3 or VEGF revealed that the increase triggered by the CNV lesion in the wildtype or PBS-injected mice were either almost eliminated (*fB*$^{-/-}$ mice) or significantly blunted (CR2-fH) ($P<0.01$; Rohrer et al. 2009; Fig. 4b).

CR2-fH was found to localize to lesion sites as shown by immuno-histochemistry, staining RPE/choroid flatmounts with an antibody against the CR2 component of the fusion protein (Fig. 5, CR2-fH) (Rohrer et al. 2009). CR2 immunopositive

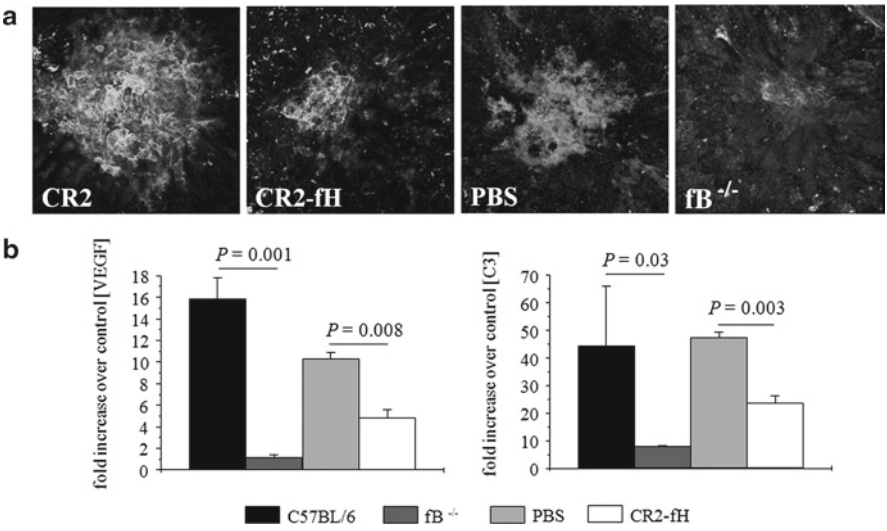

Fig. 4 CNV in CR2-fH-treated and fB-deficient mice. (**a**) CNV development was evaluated 6 days post laser photocoagulation using isolectin B4 staining. Complement factor B knockout (*fB*$^{-/-}$) mice ($n=18$) or wild type mice ($n=12$) treated with intravenous injections of PBS ($n=19$), CR2 (133 μg; $n=9$) or CR2-fH (250 μg; $n=11$) on days 0, 2 and 4 post laser photocoagulation. Isolectin B4 staining intensity is reduced in CR2-fH-treated and *fB*$^{-/-}$ animals compared to PBS and CR2-treated controls. (**b**) Analysis of VEGF and C3 mRNA expression in CNV based on quantitative RT-PCR of RPE-choroid samples removed 1 day post laser photocoagulation from wild type and *fB*$^{-/-}$, and wild type mice treated with PBS or CR2-fH. VEGF and C3 mRNA expression was significantly decreased when CAP activation was eliminated (*fB*$^{-/-}$) or inhibited (CR2-fH) (data from Rohrer et al. 2009)

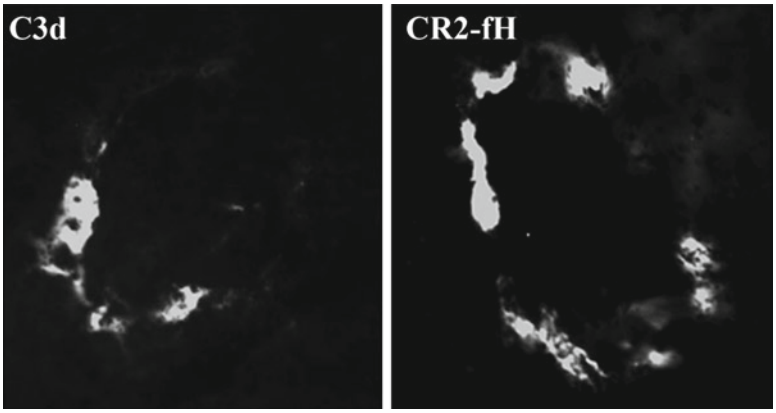

Fig. 5 CR2-fH and C3d deposition in the CNV lesion. Animals were injected with a therapeutic dose of CR2-fH (250 μg) on day 3 post laser photocoagulation, and eyes collected 24 h later. Whole-mount immunofluorescent microscopy revealed the presence of CR2-positive material in the CR2-fH-injected eyes. C3d deposition was revealed by immunohistochemistry using a C3d antibody directly conjugated to FITC in flatmounts of the RPE-choroid. Preferential labeling at the edge of the lesion was revealed using both markers (data from Rohrer et al. 2009)

staining due to deposition of CR2-fH was detected in particular at the edge of the CNV lesion in CR2-fH-treated mice (Rohrer et al. 2009). C3d deposition and CR2-fH binding show a similar pattern (Fig. 5, C3d), both occurring at the edge of the lesion. It is tempting to speculate that binding may occur on the injured RPE cells on the edge of the lesion, enlarging the area of damage by cell lysis; however, additional experiments will be needed to confirm this hypothesis.

Finally, we were able to show that we can delay the onset of treatment and hence prove efficacy in a more therapeutically relevant setting. Delaying the initiation of CR2-fH treatment until day 3 after laser injury, which represents the time of peak expression of C3, also significantly reduced the amount of CNV ($P=0.002$) and preserved retina function ($P=0.05$) (data not shown). Even delaying treatment beyond the peak of C3 mRNA expression and C3 deposition reduced the amount of CNV ($P=0.02$) (data not shown) (Rohrer et al. 2009). Taken together, CR2-fH fusion-protein injections via tail vein resulted in targeting to the eye. It inhibited CNV development when delivered acutely, and in a therapeutically relevant setting. Finally, CR2-fH was effective, based on our histological, electrophysiological and molecular readouts.

5 Summary

In summary, RPE deterioration and CNV development were both found to require activation of CAP; and specific AP inhibition reduced loss of RPE integrity, reduced VEGF secretion and blunted angiogenesis in CNV. In both the in vivo and

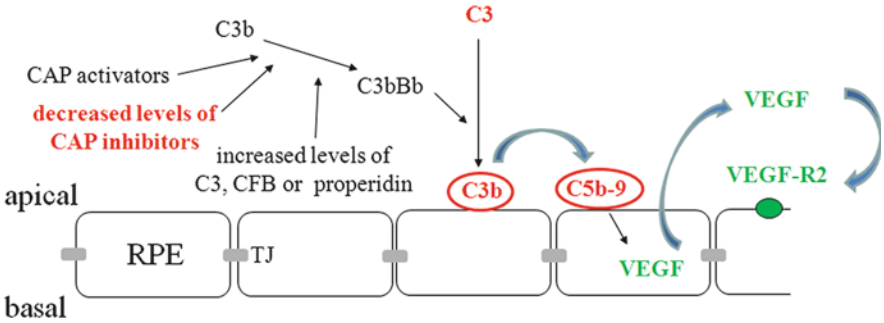

Fig. 6 Dual Hit hypothesis in RPE damage. Oxidative stress reduces the levels of membrane-bound complement inhibitors (CD55 and CD59) and hence sensitizes the RPE to complement attack. However, rather than leading to an increase in RPE cell lysis, increased CAP leads to an increase in sublytic MAC activation, which results in increased VEGF release. VEGF acting through VEGF-R2 receptors, impairs RPE function (TER breakdown and impairment of barrier function) and facilitates choroidal neovascularization

in vitro model of RPE damage, two molecular events known to be involved in AMD, complement activation and VEGF production, were confirmed (Fig. 6). We believe that the data from RPE monolayer studies, which suggest that oxidative stress reduces endogenous complement inhibitors and therefore makes the RPE susceptible to complement attack, are intriguing. This is particularly true since this concept is supported by epidemiologic and genetic data on AMD. Taken together these data may open new avenues for AMD treatment strategies.

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Chapter 11
Complement Depletion with Humanized Cobra Venom Factor in a Mouse Model of Age-Related Macular Degeneration

David C. Fritzinger, Robin Dean, Carol Meschter, Katina Wong, Roman Halter, Jürgen Borlak, William D. St. John, and Carl-Wilhelm Vogel

Abstract The effect of complement depletion with humanized cobra venom factor (CVF) on retinal lesion development/neovascularization was determined in a mouse model of wet age-related macular degeneration (AMD). Mice were treated with the humanized CVF protein HC3-1496 prior to, and once daily for 28 days after laser coagulation surgery of the retina. CVF transgenic mice exhibiting permanently low levels of serum complement activity and PBS-treated mice served as positive and negative controls, respectively. Fluorescein isothiocyanate (FITC)-dextran funduscopy after laser surgery indicated the presence of lesions in all mice that underwent laser surgery. In HC3-1496-treated mice as well as CVF transgenic mice smaller lesions were seen after 8 days. Measurement of lesion sizes by histopathological examination of eyes after 28 days revealed a significant reduction of lesion area and volume in both HC3-1496-treated animals and CVF transgenic animals compared to PBS-treated control animals. Systemic complement depletion with a complement depletor, such as the humanized CVF protein HC3-1496, represents a promising therapeutic concept for patients with wet AMD.

1 Introduction

Age-related macular degeneration (AMD)[1] is the most common cause of irreversible blindness in developed nations (Klein et al. 2004; van Leeuwen et al. 2003). The etiology of AMD is unknown although a variety of causative agents or pathological mechanisms have been proposed including infection, smoking, atrophy of the retinal pigmented epithelium (RPE), choroidal ischemia, and immune complex deposition,

[1]Abbreviations: *AMD* age-related macular degeneration, *CVF* cobra venom factor, *FITC* fluorescein isothiocyanate, *MAC* membrane attack complex, *RPE* retinal pigmented epithelium.

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to name a few (de Jong 2006; Haddad et al. 2006). Whatever the cause, the disease is characterized by local chronic inflammation in the RPE-choroid complex. The hallmark of the pathological changes is the formation of drusen, extracellular deposits that accumulate between the RPE and Bruch's membrane.

The complement system has been implicated as an important mechanism in the pathogenesis and progression of AMD. Multiple complement proteins, especially those of the alternative and membrane attack pathways, have been shown to be present in drusen (Ding et al. 2009; and references therein). More recent studies indicate that complement activation is not only a mere consequence of the disease process, but also actively contributes to disease progression. Sequence variants of complement proteins factor H, factor B, C2, and C3 have been shown to be predisposing for AMD (Edwards et al. 2005; Gold et al. 2006; Hageman et al. 2005; Haines et al. 2005; Klein et al. 2005; Maller et al. 2007; Yates et al. 2007) while others appear to be protective (Gold et al. 2006; Hageman et al. 2005). A relatively common variant of factor H with a tyrosine to histidine change at amino acid 402 (Y402H) is particularly strongly associated with AMD development (Edwards et al. 2005; Haines et al. 2005; Klein et al. 2005). Residue 402 is located within the binding sites for C-reactive protein and heparin. The Y402H variant of factor H exhibits reduced binding to these two proteins, a reduced affinity for complement component C3b, and hence a reduced ability to inhibit complement activation. These altered properties of Y402H result in continued complement activation with release of the proinflammatory anaphylatoxins C3a and C5a and formation of the membrane attack complex (MAC). Advanced AMD is characterized by choroidal neovascularization associated with the exudative or wet type of AMD which is responsible for the disabling loss of central vision [(Ding et al. 2009 and references therein) and references therein]. In a mouse model of choroidal neovascularization induced by laser photocoagulation of Bruch's membrane, complement has also been shown to play an important pathogenetic role in the neovascularization process (Bora et al. 2005).

Cobra venom factor (CVF) is the complement-activating protein in cobra venom which is highly homologous in structure and function to complement component C3 (de Bruijn and Fey 1985; Fritzinger et al. 1994; Fritzinger et al. 1992; Vogel 1991; Vogel and Fritzinger 2010). The three-dimensional domain structures of C3 and CVF are highly homologous (Janssen et al. 2006, 2009; Krishnan et al. 2009; Wiesmann et al. 2006). CVF forms a bimolecular complex with factor B. Factor B is then activated by factor D to form CVF,Bb, the so-called C3/C5 convertase of the alternative pathway, just like C3b, the activated form of C3. The convertase formed with C3b both decays rapidly into C3b and Bb and is subject to rapid inactivation by the regulatory proteins factors H and I. In contrast, the convertase formed with CVF is physico-chemically stable and resistant to the regulatory actions of factors H and I. As a consequence, CVF forms a stable convertase in serum, resulting in continuous activation of C3 and C5 and, ultimately, depletion of serum complement activity. In the prior work, the region responsible to forming a stable convertase was localized to the very C-terminal region of the CVF β-chain, which is homologous to the C-terminus of the C3 α-chain (Fritzinger et al. 2003, 2004; Wehrhahn et al. 2000). Subsequently, production of human C3 derivatives exhibiting both formation

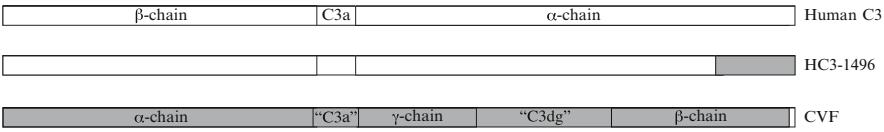

Fig. 1 Schematic representation of the chain structure of the pro-protein forms of human C3, CVF, and the humanized CVF protein HC3-1496

of physico-chemically stable convertases with factor B and significant resistance to factors H and I was achieved by replacing the C-terminal portion of the C3 α-chain with the homologous sequence from the CVF β-chain (Fritzinger et al. 2008a, 2009; Hew et al. 2004). These human C3/CVF hybrid proteins are human C3 derivatives exhibiting the core CVF function of being able to deplete serum complement activity, and are referred to as humanized CVF (Vogel and Fritzinger 2010).

The human C3/CVF hybrid protein used in this study, HC3-1496, is a humanized CVF in which the 168 C-terminal amino acid residues of C3 were replaced with the corresponding sequence from CVF (Fig. 1). Even in the 168 amino acid long exchanged region, CVF and human C3 share 43.4% sequence identity; and the overall sequence identity of human C3 and HC3-1496 is 96.3%. Humanized CVF is an experimental therapeutic for complement depletion in disease states and clinical situations where complement activation is implicated in the pathogenesis (Vogel and Fritzinger 2010). Complement depletion with humanized CVF has been shown to be safe (Fritzinger et al. 2008a, b; Gorsuch et al. 2009; Wang et al. 2009) and effective in several animal models of disease including collagen-induced arthritis (Fritzinger et al. 2008a), myocardial infarction/reperfusion injury (Gorsuch et al. 2009), and monoclonal antibody therapy of lymphoma (Wang et al. 2009). An additional fortuitous property of humanized CVF is that the convertase formed is devoid of C5-cleaving activity, thereby preventing the release of the highly pro-inflammatory C5a anaphylatoxin (Fritzinger et al. 2008b, 2009).

Given the increasing evidence of the important role of the complement system in the pathogenesis of AMD, several investigators are studying the possible therapeutic benefits of complement inhibitors in AMD (Francois et al. 2009; Iwata et al. 2009; Patel and Team 2009; Rohrer et al. 2009). Here we report initial results of a study in which complement was depleted with HC3-1496, a humanized form of CVF, as an experimental therapeutic concept in a mouse model of wet AMD.

2 Materials and Methods

2.1 Mice

C57Bl/6 mice, 23–25 g each, were obtained from Simonsen Laboratories, Inc. (Gilroy, CA).

2.2 CVF Transgenic Mice

The generation of transgenic mice constitutively expressing CVF in a BALB/c background has been described (Andrä et al. 2002). Subsequently, the CVF transgenic mice were converted into the C57Bl/6 background and maintained as hemizygotes in the transgenic animal facility of the Fraunhofer Institute for Toxicology and Experimental Medicine in Hannover, Germany, without any special housing conditions for immunocompromised mice. The CVF transgenic mice in the C57Bl/6 background exhibited average serum complement levels of approximately 30% compared to wild-type C57Bl/6 mice, which is slightly higher than was observed in CVF transgenic mice in the BALB/c background.

2.3 Animal Study

The C57Bl/6 mice were randomly assigned to either Group 1 (PBS control group), or Group 2 (HC3-1496 experimental group). The CVF transgenic mice constituted Group 3. On Day 0, all three groups were subjected to laser coagulation surgery of the retina. Animals of the HC3-1496 experimental group (Group 2) were injected i.p. with a dose of 25 µg/kg of HC3-1496 in PBS at a dose volume of 10 mL/kg PBS approximately 2–3 h prior to laser surgery on Day 0, and once daily thereafter for 28 days. Control animals (Group 1) received daily injections of PBS at the same time, frequency, duration, and dose volume as the HC3-1496 group. The CVF transgenic mice (Group 3) did not receive any injections.

Following laser surgery on Day 0, fluorescein isothiocyanate (FITC)-dextran funduscopy was performed to assure the presence of lesions in the retinal vasculature after the laser procedure. FITC-dextran funduscopy was repeated on Day 8.

The total number of animals in each group at the start of the study was eight (Groups 1 and 3) or nine (Group 2). Additional untreated and transgenic mice were subjected to laser coagulation surgery to provide for spare animals to replace study animals exhibiting procedure-related mortality during the first 24 h from anesthesia and laser surgery. One replacement animals in Group 2 did not receive HC3-1496 before laser surgery. An animal in Group 3 was found dead on Day 3 and not replaced. The final number of animal per group at necropsy on day 28 were: $n=8$ for Groups 1 and 2, and $n=5$ for Group 3.

After laser surgery, the mice were housed in individual cages. The mice were observed daily for any signs of morbidity. Body weights were recorded prior to the initial dose and laser surgery, once weekly thereafter, and at the end of the study. On Day 28, approximately four hours after the final dose of HC3-1496, animals were anesthetized and a terminal cardiocentesis was preformed. Blood was collected and serum was obtained for measurement of serum hemolytic complement activity (CH50). The right eyes (with optic nerves attached) were collected and fixed in modified Davison's solution for subsequent histopathological evaluation of retinal lesions.

The animal study was conducted in accordance with a research proposal approved by the Institutional Animal Care and Use Committee.

2.4 Humanized CVF

The humanized CVF protein HC3-1496 was prepared essentially as described (Fritzinger et al. 2009). The protein was produced in S2 *Drosophila* cells and purified to greater than 90% homogeneity. The recombinant HC3-1496 protein was a mixture of C3-like and C3b-like forms (Fritzinger et al. 2009).

2.5 Laser Coagulation Surgery

Laser coagulation surgery on the retina was performed using a modified slit lamp system as described (Kaplan et al. 1999; Miller et al. 1990). Animals were briefly anesthetized by a subcutaneous injection of ketamin:acepromazine at 80:1 mg/kg. Pupils were dilated with topically administered 1% tropicamide. Cover slips were used to flatten the cornea to aid in aiming of the laser beam. Laser coagulation surgery was performed with an Iridex Diovet diode laser, delivering 810 nm wavelength energy (90–120 mW, 50–75 m/s duration, 75 μm spot size). Three lesions were applied at approximately 9, 12, and 3 o'clock positions, 2–3 disk diameters from the optic nerve. Rupture of the Bruch's membrane was observed by bubble formation which occurred immediately at the site of photocoagulation.

2.6 Funduscopy

To visualize the retinal vasculature, mice were briefly anesthetized (on Day 0 immediately following laser surgery, and on Day 8) and injected i.v. with 100 μL of 10% FITC-dextran. Photographs of the retina were taken using a Kowa small animal fundus camera.

2.7 Histopathology

Eyes were fixed in modified Davison's solution for 12–24 h and then transferred to 10% neutral buffered formalin to complete fixation prior to processing, paraffin embedding, and sectioning. Eyes were orientated sagittally, and sectioned (10–15 serial sections at 3–5 μm through the central part of the eye, including the retinal-optic nerve region). Between 4 and 6 sections were placed on one slide. Tissue was stained with hematoxylin and eosin, and evaluated by light microscopy. The lesions (the lesion closest to the burn site and one level before and after) were measured (length and width), and two estimates of lesion size were calculated from measurements: lesion triangular area and conical volume. An analysis of variance was performed on the triangular area and conical volume data from the

three groups, as well as pair-wise comparisons using the SAS software package (Snedecor and Cochran 1989).

2.8 Hemolytic Complement Activity

The hemolytic complement activity (CH50) in mouse serum was determined as described previously (Wang et al. 2009).

3 Results

Retinal lesions with choroidal neovascularization similar to those seen in AMD were induced in mice by laser photocoagulation of the Bruch's membrane. The presence of retinal lesions was confirmed immediately after laser surgery on Day 0 in all animals using FITC-dextran funduscopy (Fig. 2). The lesions were again assessed by FITC-dextran funduscopy on Day 8. Notwithstanding variation from animal to animal, the lesions in the HC3-1496 treatment group and in the CVF transgenic mice appeared smaller than the lesions in the PBS control group, particularly on Day 8 (Fig. 2).

To assess the effect of complement depletion on laser-induced damage to the retina and subsequent choroidal neovascularzation, mice were depleted of their serum complement activity by injection of 25 μg/kg HC3-1496 on Day 0 prior to laser coagulation surgery and daily thereafter for 28 days. A second experimental group consisted of CVF transgenic mice which constitutively express CVF and exhibit low serum complement activity. The control group consisted of mice injected daily with PBS. Animals that died within the first day of laser surgery were replaced with laser-treated spare animals. One animal never recuperated and died on Day 3 and was not replaced. All animals surviving the initial trauma appeared healthy throughout the study and exhibited stable body weights. No treatment-related toxicity was observed in the HC3-1496 treatment group.

On Day 28, the animals were sacrificed, and the retinal lesions were measured by histopathological examination of formalin-fixed, paraffin-embedded slide-mounted tissue sections. Two different estimates of the retinal lesion size were calculated from the histological lesion measurements, a triangular area and a conical volume. Figure. 3 demonstrates that both lesion area and lesion volume were significantly smaller in both the HC3-1496 treatment group and the CVF transgenic mice compared to the PBS control group.

On Day 28, the mean serum complement activity of the HC3-1496 treated mice was approximately 70% compared to wild-type C57Bl/6 mice. However, there was significant mouse to mouse variation, from approximately 30% complement activity to 100% complement activity.

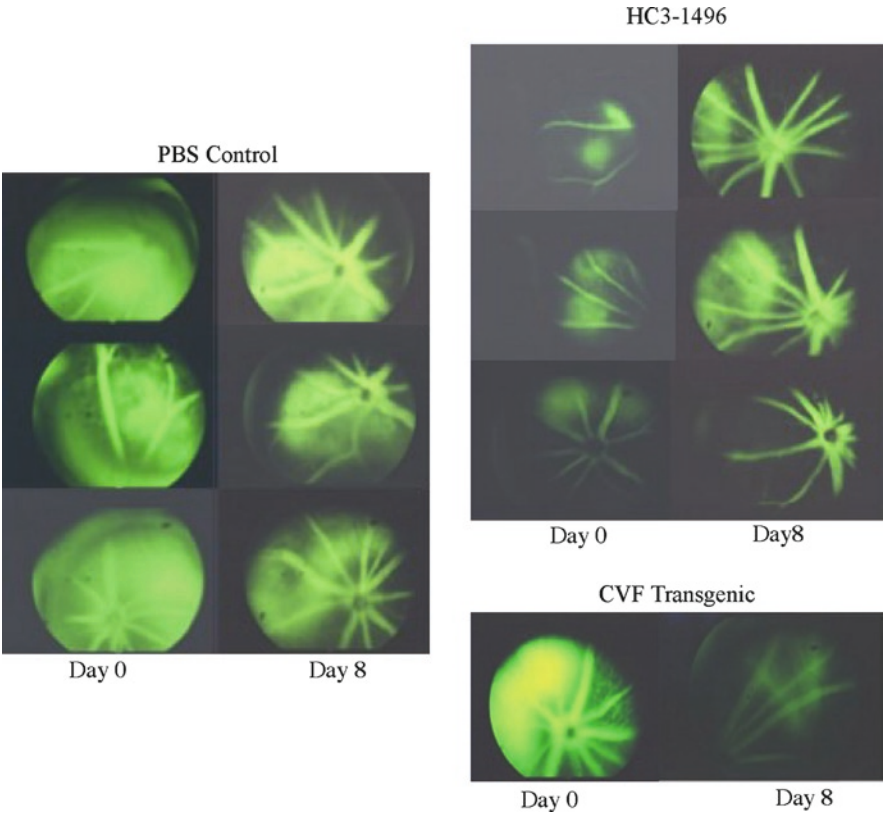

Fig. 2 FITC-dextran funduscopy immediately after laser coagulation surgery (Day 0) and on Day 8 in mice treated with HC3-1496, CVF transgenic mice, and PBS-treated control mice

4 Discussion

Activation of the complement system has been identified as an important pathogenetic mechanism in the development of AMD. Therefore, pharmacological inhibition of complement represents a potential therapeutic approach for AMD, and wet AMD in particular, that is being studied by a number of investigators (Francois et al. 2009; Iwata et al. 2009; Patel and Team 2009; Rohrer et al. 2009). Most experimental therapeutic approaches to inhibit complement activation involve the development of agents that either prevent the activation of a given complement component or inhibit the biological effects of an activated component (Holers and Thurman 2004; Morgan and Harris 2003; Ricklin and Lambris 2007; Sahu and Lambris 2000). Our laboratory has developed a conceptually different approach, complement depletion (by exhaustive complement activation) based on the activity of a naturally occurring protein, CVF. Temporary, prolonged, or episodical elimination of complement will eliminate the harmful effects of complement activation in a

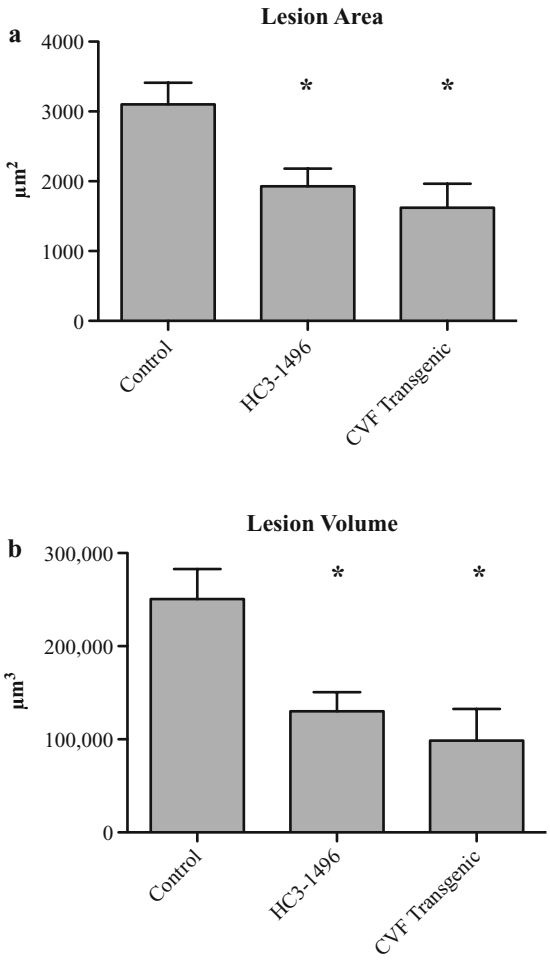

Fig. 3 Lesion area and lesion volume as determined by histopathological examination of fixated eyes on Day 28 in mice treated with HC3-1496, CVF transgenic mice, and PBS-treated control mice. Both lesion area and lesion volume are significantly smaller in HC3-1496-treated mice and CVF transgenic mice compared to PBS control mice. Lesion area and volume are not significantly different between HC3-1496-treated mice and CVF transgenic mice

pathological situation, regardless of how potent the signal for complement activation is (Vogel and Fritzinger, 2010). We have developed a humanized form of CVF which is a derivative of human complement protein C3. Humanized CVF exhibits the core function of CVF of forming a physico-chemically stable convertase which is also resistant to rapid inactivation of Factor H and I, causing complement depletion (Fritzinger et al. 2008a, 2009; Vogel and Fritzinger 2007).

In a murine model of wet AMD, which is based on laser-induced lesioning of Bruch's membrane and the RPE resulting in choroidal neovascularization, we show

that treatment with the humanized CVF protein HC3-1496 significantly reduces the extent of retinal lesions. Complement depletion prior to laser surgery may reduce the size of the initial lesions as evaluated by FITC-dextran funduscopy. Both the area and volume of retinal lesions as measured histopathologically after 28 days of HC3-1496 treatment were significantly smaller compared to complement-sufficient PBS-treated control animals. Lesion area and volume in animals treated with HC3-1496 were indistinguishable from lesion area and volume in CVF transgenic mice which permanently exhibit low serum complement levels (Andrä et al. 2002). Our results are consistent with a previous study that showed that the extent of laser-induced choroidal neovascularazation both in mice complement-depleted with CVF as well as in C3-deficient mice was markedly reduced, with attenuated tissue deposition of both C3b and the MAC (Bora et al. 2005).

It is important to point out that the dose of HC3-1496 used in this study (25 μg/kg) is very low. Complement depletion with 25 μg/kg of CVF in mice is so short-lived that serum complement levels return to normal within 24 h after CVF administration (Vogel 1991; Vogel and Fritzinger 2010). Whereas humanized CVF exerts its complement-depleting action as rapidly as CVF, the resulting complement depletion is not as long lasting as that induced by CVF (Fritzinger et al. 2008a; Vogel and Fritzinger 2007). Our study did not allow us to obtain detailed kinetic information of complement levels throughout the 28 day period. However, the mean serum complement activity in HC3-1496-treated mice was 70%, with significant variation from animal to animal. This variation may reflect the time difference from the last dose of HC3-1496 administration and the time of sacrifice of individual animals, given the low dose of HC3-1496 used. Whereas more detailed analyses of serum complement levels during repeated administration of a low dose of a complement depletor such as HC3-1496 are warranted, our results demonstrate that repeated administration of HC3-1496 at a dose not causing significant complement depletion over an extended period of time is sufficient to significantly reduce the retinal lesions. The observed beneficial effect of treatment with the low dose of HC3-1496 was indistinguishable from the state of permanent complement depletion in the CVF transgenic animals. This result indicates that only a limited reduction of serum C3 levels or serum complement activity is sufficient to counteract the pathogenetic activity of complement in AMD (and possibly other diseases).

The results of this study demonstrate that systemic complement depletion either by administration of humanized CVF or by constitutive expression of CVF in the transgenic animals had a significant, favorable affect on AMD progression in this murine model of AMD. Whereas this is consistent with the results of an earlier study using either CVF for complement depletion or C3-deficient mice (Bora et al. 2005), it demonstrates that systemic complement components present in the circulation are involved in the pathogenesis, and that systemic complement depletion effectively reduces tissue damage and neovascularization. Whereas local production of complement components in the eye, particularly of the alternative pathway, has been demonstrated (Anderson et al. 2009), our study confirms that local production of complement components is not responsible for the complement-mediated pathogenesis in AMD.

In conclusion, this pilot study of complement depletion in a murine model of wet AMD suggests that systemic complement depletion with humanized CVF represents a promising therapeutic approach for patients with wet AMD. The study results further suggest that only a relatively small extent of complement depletion appears to be sufficient to produce a beneficial effect.

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Disclosure D. Fritzinger, W. St. John, and C.-W. Vogel have a financial interest in Incode Biopharmaceutics, Inc., San Diego, CA.

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