Age-Related Macular Degeneration: The Molecular Link between Oxidative Damage, Tissue-Specific Inflammation and Outer Retinal Disease
The Proctor Lecture

Joe G. Hollyfield

Age-related macular degeneration (AMD) is the third leading cause of vision loss worldwide and the most common form of legal blindness in the elderly.1 Millions of individuals who are older than 50 years in Europe and North America have AMD, and it is estimated that more than 300,000 new cases are diagnosed annually.2,3 During aging, focal deposits of debris can accumulate below the retinal pigment epithelium (RPE) that are recognized in an eye examination as drusen. Clinicians have also recognized that drusen in the macula are early stages in the AMD disease process. Drusen in the macula and the size and area covered by these deposits are considered risk indicators for later development of the blindending-stage forms of AMD.4–6 Geographic atrophy is the end-stage of the dry form, characterized by the slow, progressive loss of the RPE.7 Photoreceptor function is lost in regions subtended by RPE atrophy. When geographic atrophy involves the RPE below the fovea, foveal photoreceptors lose function and degenerate, causing foveal blindness. Choroidal neovascularization characterizes the wet form of AMD. When new blood vessels growing from the choroid break through Bruch’s membrane and the RPE, they can hemorrhage, causing a pool of blood to accumulate between the RPE and foveal photoreceptors resulting in acute loss of vision.4,5

Initial studies on AMD by my colleagues and myself focused on understanding the composition and distribution of drusen proteins.8–13 More than 120 different proteins were identified in isolated drusen. Several laboratories have made significant contributions to the understanding of drusen composition in recent years, and a consistent finding is that many complement pathway proteins are present in drusen, suggesting that inflammation is a part of the AMD disease process.9,14–18

Epidemiologic studies of elderly twins pointed to the likelihood that genetic factors have a role in AMD.19–22 Recent reports indicate that mutations/polymorphisms in genes coding for the alternative complement pathway regulator (factor H and factor H-related proteins) and complement pathway proteins (complement component C2, C3, and factor B) are present in more than 50% of patients with AMD. Collectively, these studies strongly indicate that AMD has a genetic component and that inflammation plays an important role in the pathology of AMD.23–26

The complement system is critical for inflammation and immune responses. Soluble complement proteins are present in the blood in precursor forms and require activation to achieve their specific physiological roles. Activated complement has diverse functions, including the initiation of inflammation, recruitment of leukocytes, clearance of immune complexes, neutralization of pathogens, regulation of antibody responses, and disruption of cell membranes. The complement cascade can be activated by three initiating pathways. The classic activation pathway depends on assembly of complement factors at sites of antigen–antibody complexes. The lectin pathway is initiated by mannan-binding lectin on pathogen surfaces. Activation of the alternative pathway is triggered by the spontaneous hydrolysis (“tickover”) of the third complement component (C3), allowing binding to factor B and factor D. Regardless of the pathway, activation leads to the cleavage of C3, which generates the smaller, proinflammatory C3a fragment and the larger C3b fragment. C3b interacts with other activated proteins to form the important convertases required for the terminal part of the complement cascade that culminates in the assembly of the C5b-C9 membrane attack complex (MAC) and cell lysis. Most of the complement pathway proteins are found in drusen and sub-RPE deposits, including C5b-9, indicating that the MAC has been assembled.9,14–18

Although the data indicating that the complement pathway is involved in AMD are unequivocal, it is not known how systemic changes caused by polymorphisms in complement pathway genes could result in a localized disease in the outer retina. I reasoned that the highly targeted lesions of the RPE and photoreceptors of the macula must be directed by a specific inflammatory signal generated and released by these tissues. An early hint of a potential inflammatory signal that could come from these tissues was the finding that many proteins found in drusen were not normal (native), but were covalently cross-linked into high-molecular-weight aggregates, modifications that can be caused by reactive lipid and carbohydrate oxidation products. Many of the drusen proteins were covalently modified with adducts, including advanced glycation end products and pyrrole adducts generated from the oxidative damage of docosahexaenoic acid (DHA).9 Carboxyethylpyrrole (CEP) adducts were identified on albumin, pyruvate kinase, glutathione S-transferase, and several other drusen proteins.8 In addition, it was found that these CEP adducts were more prevalent in AMD donor eyes than in RPE/Bruch’s membrane/choroid tissues from normal age-matched donor tissue.8 Plasma samples from patients with AMD contained 40% higher levels of CEP-adducted protein and autoantibodies to CEP than were present in plasma of healthy age-matched donors without AMD.54–57 The presence of these oxidation fragments derived from DHA are of particular interest because of the long-recognized association of AMD with oxidative damage.54–57 The findings that smoking is a risk factor for AMD54,58,59 and anti-oxidant vitamins and zinc reduce the risk of vision loss in AMD...
also support the notion that oxidative damage plays a fundamental role in this disease. Could this CEP adduct, derived specifically from oxidative damage to DHA, be the inflammatory signal from the outer retina that initiates AMD?

DHA is the most oxidizable fatty acid in the body and is highly concentrated in the membrane-rich photoreceptor outer segment and in the RPE. High levels of oxygen delivered to these tissues by the choriocapillaris, coupled with the intense focus of environmental light on the macula provides a highly permissive environment for the generation of reactive oxygen that can damage DHA. Once generated, these oxidation fragments condense with amino acid groups in proteins (i.e., Cys, Lys, and His) to generate Michael, Schiff-base, and pyrrole adducts. Although DHA is only one of many sources of lipid-derived protein adducts, its extremely high concentration in the outer retina makes this fatty acid a likely source of adducts in this tissue. Other polyunsaturated fatty acids, their phospholipids, and cholesterol ester derivatives can also generate a complex mixture of oxidation products that are capable of reacting with protein. Pyrrole adducts are an important class of lipid-derived protein modifications that include CEP from DHA; carboxypropylpyrrole (CPP) from arachidonic acid; and carboxyheptapyrrole (CHP) from linolenic acid.

Interest was focused on CEP because of the following considerations: (1) CEP is a novel adduct that is generated only by oxidative damage to DHA; (2) DHA, although present in every cell in the body, is more concentrated in the outer retina in photoreceptors and RPE than in any other cell in the body; (3) high oxygen levels and light in the outer retina provide a permissive environment for oxidative damage to DHA; (4) CEP adducts are more abundant in the outer retina in AMD donor eyes than in normal age-matched control eyes; and (5) CEP adducts and autoantibodies to CEP are more abundant in the serum of patients with AMD than in normal age-matched control subjects. With these considerations, a hypothesis was developed as to the identity of the inflammatory signal generated in the outer retina and why individuals with mutations/polymorphisms in complement pathway genes might be more susceptible to developing AMD: CEP adducts are slowly generated during aging in the outer retina. Some of the CEP precursor fragments or CEP-adducted proteins are deposited in Bruch’s membrane and in the debris accumulating below the RPE. CEP adducts are antigenic, and antibodies against CEP are produced by the immune system. In individuals with normal protective mechanisms against oxidative damage, only small amounts of these adducts are generated. In contrast, more adducts are likely to be produced in individuals who have compromised protection against oxidative damage (e.g., smoking, superoxide dismutase dysfunction, etc.).

When an inflammatory response is mounted against CEP adducts in an individual with an altered ability to regulate the complement activation, the possibility of end-stage AMD is greatly enhanced. To test some of the ideas in this hypothesis, a series of immunization experiments in normal mice was set up to determine whether CEP adducts may be an inflammatory signal generated in the outer retina that leads to complement-mediated disease during aging. Although the photoreceptors are behind the blood retinal barrier, and apical border of the RPE is also inside the blood retinal barrier, the basolateral border interacting with Bruch’s membrane is not. It is likely that much of the CEP adducts generated by oxidative damage in photoreceptors and RPE are released slowly during aging for interaction with the immune system. By systemically immunizing mice with a CEP-adducted protein I expected to sensitize mice to endogenously generated CEP. I predicted that as these sensitized mice aged, endogenous CEP adducts generated preferentially by these cells would result in a more aggressive immune response and some pathologic features similar to AMD may appear.

As a carrier for the CEP hapten I used mouse serum albumin (MSA) and chemically modified it to generate CEP-MSA. Aliquots of CEP-MSA were emulsified in complete Freund’s adjuvant (CFA) and C57BL/6 mice were immunized after conventional immunization procedures. Two immunization protocols were followed: a “short-term” protocol with three immunizations during a 3-month period; and a “long-term” protocol with a single immunization and maintenance of the mice for 12 to 14 months. Spleen, lymph nodes, eyes, kidney, brain, testes, and blood were sampled for immunologic and/or histologic analysis after euthanization. Control immunizations used were nonadducted MSA or complete Freund’s adjuvant (CFA) applied in the same sequence as CEP-MSA.

The sera from these mice were evaluated for CEP antibodies by ELISA and it was found that circulating anti-CEP antibody levels were six to eight times higher in the CEP-MSA immunized mice than in the naive and control mice immunized with MSA or CFA. For pathology, eyes taken at the end of the experimental period were prepared for detailed microscopic analysis by procedures described previously. In eyes from the CEP-MSA mice in the short-term immunization protocol, dramatic lesions were present that primarily involved the RPE. These changes included vesiculation, hypertrophy, pyknosis, and lysis of RPE cells. In some areas, only single RPE cells were involved, whereas in other areas stretches of the RPE over 2 to 10 cell expanses showed these pathologic changes. More extensive degeneration was occasionally observed involving areas where the entire RPE was absent, producing focal areas similar to that observed when the RPE is lost in geographic atrophy. Photoreceptors under areas of RPE atrophy remained but were highly edematous and swollen. The amount of degeneration in these experiments was scored by counting the number of individual lesions in each of three or four sections examined throughout the retina from the dorsal to the ventral ciliary margin in each eye. When the degree of pathology to the antibody titer in these animals was compared, a close correlation between the CEP-antibody titer and the severity of outer retina disease was noted.

Is the complement system involved in the generation of the disease observed in the RPE? Complement component 3 (C3) is a key complement protein that must be processed for complement activation through classic, lectin, or alternative arms. To determine whether complement deposition occurred in these CEP-MSA immunized mice, immunohistochemical analysis was performed to localize of C3d, a degradation fragment of C3 that is deposited in tissues where complement is activated. Frozen sections were analyzed from CEP-MSA immunized and control mice after staining with an anti-C3d antibody. A strong C3d signal was present in Bruch’s membrane below the RPE in the mice receiving CEP-MSA immunization (Fig. 2a), and except for minor fluorescence occasionally seen in mice immunized with the nonadducted MSA (Fig. 2b), the controls were free of C3d immunoreactivity (Fig. 2c).

Histology of the outer retina in mice immunized with CEP-MSA in the long-term protocol and examined 12 to 14 months later revealed an accumulation of sub-RPE deposits. Although these deposits are evident at the resolution of light microscopy, their structural features are more striking when viewed with electron microscopy. Figure 3 presents a comparison of the RPE/Bruch’s membrane/choriocapillaris interface from age-matched naive mouse (Fig. 3a) and a mouse immunized with...
CEP-MSA 1 year before death. In the CEP-MSA immunized animal, an extensive buildup of flocculent material is present between the greatly expanded basal infoldings of the RPE. Some of this debris shows distinct banding patterns with a periodicity characteristic of “long-spaced collagen.” Bruch’s membrane is also greatly swollen. Choroidal neovascularization was not observed in any of the animals, suggesting that the disease generated is a model for the dry-type of AMD. Sub-RPE material did not develop in control subjects, although increased thickening of RPE basal infoldings was evident with aging.

These data establish the proof of concept that CEP-MSA immunized C57BL/6 mice develop immune responses to CEP-MSA immunization that result in targeted RPE changes in the outer retina. The correlation between the level of anti-CEP antibody titer and the degree of disease suggests that activation may be mediated through the classic arm of the complement activation pathway, leading to the deposition and processing of C3 in Bruch’s membrane. The focal loss of RPE observed resembles geographic atrophy, one of the end-stage conditions in human AMD. Although it is unlikely that CEP is the only

FIGURE 1. Pathology of the RPE in CEP-MSA–immunized mice according to the short-term protocol. The RPE is oriented in each micrograph with the apical surface along the top border of each image and Bruch’s membrane along the bottom border. (a) Vesiculation of the RPE. Four distinct areas of cytoplasm in several adjacent RPE cells are evident. (b) The dark staining condition of pyknosis of the RPE on the left and hypertrophy of the RPE on the right. (c, d) Lysis of the RPE. A single RPE cell is undergoing lysis in (c), whereas in (d) several RPE cells are involved. (e) Three adjacent pyknotic (darkly staining) RPE cells. The specificity of these changes in the RPE after CEP-MSA immunization suggests that the RPE is targeted by the immune system, because it is a site of CEP adduct generation. Each image was photographed and displayed at the same magnification. Bar, 10 μm.

FIGURE 2. C3d localization in Bruch’s membrane in the short-term immunization mice. (a) A confocal image from a CEP-MSA–immunized mouse shows bright green fluorescence of the C3d-FITC probe along Bruch’s membrane, located along the horizontal band (arrows) on the right side of each image. (b) A small amount of immunofluorescence is present in this specimen from a mouse immunized with MSA alone. (c) No immunofluorescence is present in Bruch’s membrane in this preparation from a mouse receiving only the adjuvant. Each section was also stained with propidium iodide, but only a small amount of nuclear fluorescence is evident because of the quenching by the highly melanized RPE and choroid in C57/Bl mice used in these studies. Each micrograph shown is photographed and imaged at the same magnification. Bar, 10 μm.

FIGURE 3. Electron micrographs of the interface between RPE/Bruch’s membrane/choriocapillaris from age-matched mice comparing the normal structure of this interface in a naïve animal (a) with the changes caused by CEP-MSA immunization (b). Mice were 15 months old when killed. The CEP-MSA immunization occurred 1 year before death. Horizontal dashed line: the central elastin lamina of Bruch’s membrane. Top and bottom brackets: thickness of the basal infoldings and Bruch’s membrane in the naïve mouse, respectively. Note the great increase in these two layers in the CEP-MSA immunized mouse. Although the membrane profiles of the basal infoldings are clearly evident in (b). These processes are greatly displaced from each other by the accumulation of flocculent, extracellular material, some of which show distinct banding pattern consistent with long-spaced collagen. Both micrographs were photographed and presented at identical magnifications. Bar, 1 μm.
inflammatory signal involved in the initiation of an immune response leading to AMD, it is clear from these studies that when this hapten is presented to normal mice in the manner described, it is sufficient to initiate a targeted attack on the RPE. This is the first demonstration that a hapten generated by the oxidative damage to DHA\(^{45}\) that is present in drusen of patients is sufficient to initiate a targeted attack on the RPE. This demonstrates that a target specific T-cell response is initiated when this hapten is presented to normal mice in the manner described. 

From the outcome of these studies, a new working model for AMD generation has emerged that integrates the idea of a tissue-specific inflammatory signal with the recent findings that polymorphisms/mutations in complement pathway genes are linked to AMD. This model is built on the concept of an interplay between the rate of inflammatory signal generation in the outer retina and autoantibody levels increase during aging, the level of these signals do not reach the disease threshold during the lifetime of the individual. In Figure 4b, the rate of inflammatory signal from the outer retina and autoantibody levels progress at the same rate as in Figure 4a, but polymorphisms/mutations in complement genes lower the disease threshold and allow these two lines to intersect and AMD to be manifest. For simplicity, Figure 4b depicts complement polymorphisms as having an effect on reducing the disease threshold. Several complement polymorphisms are described that reduce the risk for AMD, which in this model would elevate the disease threshold. In Figure 4c, changes in the systemic protection in oxidative damage, or environmental conditions that are permissive of oxidative damage result in an increase in the rate of inflammatory signal generation, changing the slope of the dashed line and allowing an early intersect with the disease threshold. Changes in the right and left ordinates should be expected to vary independently. Higher rates of inflammatory signal generation coupled with a lower disease threshold would result in an early onset of AMD. Conversely, a lower rate of inflammatory signal generation in an individual with efficient complement regulation would be reflected in the graph in Figure 4a. Figure 4d shows how the immunization of the mouse with CEP fits with this AMD disease model. Normally, CEP would be generated in the outer retina and slowly evoke an immune response that would not reach the disease threshold (similar to that shown in Fig. 4a). Systemic immunization in the mouse changes the slope of the dashed line (through the generation of the CEP antibody), which rapidly reaches the disease threshold. Complement mediated attack is directed toward the RPE in the outer retina because of the specific generation of CEP adducts in this tissue.

This mouse model for AMD provides a new resource for understanding the early interaction of inflammatory signals originating in the outer retina with the immune system that initiate AMD. Progression of this outer retinal pathology and the severity of these lesions can also be studied in mice that are missing key complement pathway genes, as a means of evaluating the relative importance of the different complement activation arms in AMD. Because the model is based on an inflammatory signal discovered in AMD eye tissues and replicates several features of AMD, it may also become a useful preclinical platform for testing the efficacy of drugs that are designed to prevent or modify the rate of pathologic progression.

**Acknowledgments**

I am deeply honored to receive the Proctor Medal from ARVO. I thank those who nominated me for this recognition and the Awards Committee and the Board of Trustees for selecting me for this distinction. I have been a member of this association and attended every ARVO meeting since 1970 and have benefited greatly from the scientific interactions and friendships that have developed with ARVO members. To be chosen for this recognition by my peers is the signal honor of my career.

I appreciate the friendship and collegiality of Robert E. Anderson, MD, PhD, and thank him for his generous introductory remarks. I met Gene at my first ARVO in 1970, and we have had a long and continuous friendship since that initial meeting. After my move to the Cullen Eye Institute at Baylor College of Medicine in 1977, we were on the same faculty, our laboratories were on the same research floor, and we were close colleagues and collaborators for 18 years before each of us relocated to other institutions.

I will always be grateful to the mentors who guided me as a student. I thank Arthur A. Johnson, my biology advisor at Hendrix College, who...
gave me the first glimpse of the excitement of academics and the possibility of a research career in biology. I thank Willie Reams, Jr, supervisor of my Master’s thesis at Louisiana State University in Baton Rouge, for introducing me to the embryo. In the laboratory of John H. Davidson at LSU, I first began to work with the frog embryo, which became the experimental animal of choice for my PhD thesis work. Special thanks go to Anton G. Jacobson at the University of Texas in Austin, who was my PhD thesis advisor. Jake demanded that each of his graduate students have every opportunity to develop and mature as independent scientists. On my first day in his laboratory, he asked me what I wanted to do for my thesis research. When I told him that I wasn’t sure, he instructed me to spend some time in the library and come back and talk with him again when I felt that I had some good ideas. To be challenged as a graduate student to formulate a thesis proposal that was unique, develop the experimental design, and work through the problems that arise during execution of the studies was for me an outstanding educational experience. I will be forever grateful to him for providing this style of graduate education. I also am pleased to acknowledge the support and guidance of the late Peter D. Nieuwkoop, Director of the Hubrecht Laboratory in Utrecht, The Netherlands, who supervised my postdoctoral studies. I came to ophthalmology research not by careful career planning, but quite by accident. My PhD thesis work was focused on lineage analysis of circulating red blood cells in the frog, Rana pipiens. During the course of these studies I discovered the rapid turnover of the red blood cell during metamorphosis. I was aware that there were changes in the chromophore of visual pigments in photoreceptors as this animal transitioned from the completely aquatic environment of the tadpole to the semiaquatic existence of the adult. Naively thinking that there may be some photoreceptor replacement during metamorphosis, I proposed to evaluate cellular changes in the retina in amphibians at metamorphosis in a Fight for Sight Postdoctoral Fellowship application. The fellowship was funded, and a few months after beginning my postdoctoral studies I received a letter from the late George G. Smerber, Director of the Ophthalmology Research Program at Columbia University College of Physicians and Surgeons in New York. In this correspondence he stated that he was, “aware of my interest in retinal development.” I was stunned by this comment, because I had not published on retinal development! I learned later that he had reviewed my fellowship application as a member of the Fight for Sight review panel. He went on to indicate that if I was, “interested in pursuing additional studies of the development of the eye” after the completion of my fellowship there might be a faculty position available at Columbia P&S. Eighteen months later, I arrived at 165th Street and Fort Washington Avenue in upper Manhattan to begin my academic career as an Assistant Professor of Anatomy (assigned to Ophthalmology), without ever having a personal interview or presenting a seminar. I am appreciative of the research support received over the years from the following public and private foundations (listed in chronological order): American Cancer Society (Louisiana Chapter), Fight For Sight, American Philosophical Society, National Eye Institute (NIH), Retina Research Foundation, Foundation Fighting Blindness, Alcon Research Institute, Wolf Family Foundation, and the Llura and Gordon Gund Foundation. I am also appreciative of the research interactions with the following pharmaceutical companies: Merck, Alcon, LuxBioscience, Genentech, Novartis, and Allergan.

The studies presented in my Proctor Lecture and described in abbreviated format here are the outcome of an ongoing collaboration between my laboratory and a number of other investigators. The early concept of the CEP adduct as a biomarker for DHA oxidative damage originated with Robert G. Salomon, PhD, and all the CEP-adducted proteins and some of the antibodies used in these studies were prepared in his laboratory. The observations that CEP adducts were present in drusen and were more abundant in AMD Bruch’s membrane/choroid samples than in age-matched control tissues were performed in collaboration with John W. Crabb, PhD. The mouse model of AMD was developed in collaboration with Rafael L. Ufret, MD, and Victor L. Perez, MD. These collaborations have been extremely fruitful, and I look forward to continued interaction with these investigators in the future. I have also had a number of discussions and received valuable advice and suggestions on some of the immunology performed or planned using this mouse model from V. Michael Holers, MD, Joshua M. Thurman, MD, and Robert B. Nussenblatt, MD, MPH, which I gratefully acknowledge. My very special and sincere thanks go to Mary E. Rayborn, my wife, friend, and partner, both in and out of the laboratory. Mary is a coauthor of many of the publications from the laboratory. She is a gifted microscopist and has generated most of the elegant histology, electron microscopy, and immunocytochemical images used in many of our publications. We have shared many adventures during our journey that now spans nearly four decades. I cannot imagine this time without her love, support, and help.

References

17. Hageman GS, Luthert PJ, Chong NH, Johnson LV, Anderson DH, Mullins RF. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch’s


