Oxidative Stress in Applied Basic Research and Clinical Practice

Robert D. Stratton
William W. Hauswirth
Thomas W. Gardner *Editors*

Studies on Retinal and Choroidal Disorders

Humana Press

Oxidative Stress in Applied Basic Research and Clinical Practice

Editor-in-Chief
Donald Armstrong

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Note from the Editor-in-Chief

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Preface

The current state of understanding the roles that oxidative and nitrosative/nitrative stress play in cellular metabolism of the retina as well as in diseases of the retina is the subject of this volume. The extreme complexity of the physiology of the retina in health and in disease has not yet been fully determined, but analysis of this complicated system has been accelerating. More mature is the analysis of the retinal physiology in the healthy retina due mainly to the physiologic similarity between humans and other animals. It is much easier and vastly less expensive to study in vitro systems and animal models than to study human subjects. To answer one prospective question to a desired degree of certainty in human disease treatment requires tens of millions of dollars and years of follow up. As a result, in vitro and animal model studies have advanced rapidly in the academic realm, while human studies lag behind, and are funded more and more by private enterprise whose principal aim is justifiably to secure government approval for a potential therapy. Because of this disperity between basic science and clinical research, there is necessarily an emphasis on basic science, but relevant clinical research is included.

The book begins with three chapters that review the etiologies of AMD, look at the direction of new treatment strategies, review the complement system in AMD, and explain oxidative stress in the pathology of AMD. Detailed explanations of oxygen stress in the lipid metabolism of the retina are given in Chaps. 4–8. Chapter 9 shows the relationship between the antioxidant system of glutathione and α-crystallins that explains the anti-apoptotic activity of the latter. The roles of the mitochondria and the endoplasmic reticulum in oxidative stress and retinal dysfunction are discussed in Chaps. 10 and 11.

The role of iron in retinal disease, the mechanisms of pathological VEGF expression, and the role of NAPDH oxidase are the subjects of Chaps. 12–14. Chapters 15–18 discuss the role of oxidative stress in oxidized lipoproteins, hepatocyte growth factor, the *Ccl2*$^{-/-}$*/Cx3cr1*$^{-/-}$ mouse model of AMD, and the systemic changes in AMD. Cerium oxide nanoparticle reduction of oxidative stress in the retina is the topic of Chap. 19.

Chapters 20 and 25 discuss the role of progenitor cells in the cause and treatment of retinal disease including AMD and diabetes. An exhaustive look at natural compounds used in the prevention and treatment of retinal disease is given in Chap. 21. Chapter 22 discusses serotonin 5-HT_{1A} receptor agonists in oxidative stress and retinal disease. Anti-VEGF treatment strategies for neovascular AMD are examined in Chaps. 23 and 24.

Nitric oxide and inducible nitric oxide synthase in retinal vascular disease are explored in Chap. 26. The effect of lipid hydroperoxide on circulating leukocytes was evaluated by an in vivo technique of acridine orange digital fluorography in Chap. 27. The role of oxidative stress in retinopathy of prematurity is discussed in Chap. 28. VEGF inhibitor-induced oxidative stress in retinal ganglion cells is examined in Chap. 29. With Chaps. 30 and 31, the book ends with a careful look at the role of carotenoids in retinal health and disease.

We thank our authors for their efforts to make this book a timely and thorough review of the advances in understanding the role of oxidative stress in health and disease of the retina. We are sure that readers will gain a better understanding of the pathophysiology and potential treatments of vascular and degenerative diseases of the retina, and hope readers will agree that the future looks bright with effective new treatments and new areas for exploration.

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Chapter 1
Review of Emerging Treatments for Age-Related Macular Degeneration

Marco A. Zarbin and Philip J. Rosenfeld

Abstract In the era of pathway-based therapy, all treatments for AMD will address some step in the pathway that leads from early to late AMD. Steps in AMD pathogenesis that appear to be good targets for drug development include the following: (1) oxidative damage, (2) lipofuscin accumulation, (3) chronic inflammation, (4) mutations in the complement pathway, (5) mitochondrial damage, (6) *Alu* RNA accumulation in RPE, and (7) BMP-4 accumulation in RPE. Steps in neovascularization that can be targeted for drug development and combination therapy include the following: (1) angiogenic factor production, (2) extracellular factor release, (3) binding of factors to extracellular receptors (and activation of intracellular signaling after receptor binding), (4) endothelial cell activation (and basement membrane degradation), (5) endothelial cell proliferation, (6) directed endothelial cell migration, (7) extracellular matrix remodeling, (8) tube formation, and (9) vascular stabilization. Combination therapy will likely supplant monotherapy as the treatment of choice because the clinical benefits will likely be superior in preventing the complications of AMD.

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1.1 Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness among persons over age 55 years in the industrialized world. Currently, approximately 1.75 million people in the USA have late-stage AMD (i.e., geographic atrophy (GA) and/or choroidal new vessels [CNVs]), and approximately seven million have drusen larger than 125 μm and thus are at relatively high risk of developing late stage disease [1]. Many treatments for early and late stage AMD are in preclinical development or in early clinical trials (Fig. 1.1). These treatments are based on seven features of AMD that seem relevant to its pathogenesis. In this chapter, we consider first a hypothesis of AMD pathogenesis that incorporates these observations and then review treatments in various stages of development in the context of this hypothesis.

1.2 Pathogenesis of AMD

Detailed consideration of the pathogenesis of AMD is beyond the scope of this chapter but has been discussed elsewhere [2, 3]. Seven features of AMD pathogenesis are considered: (1) oxidative damage, (2) lipofuscin accumulation, (3) chronic inflammation (possibly including parainflammation), (4) mutations in components

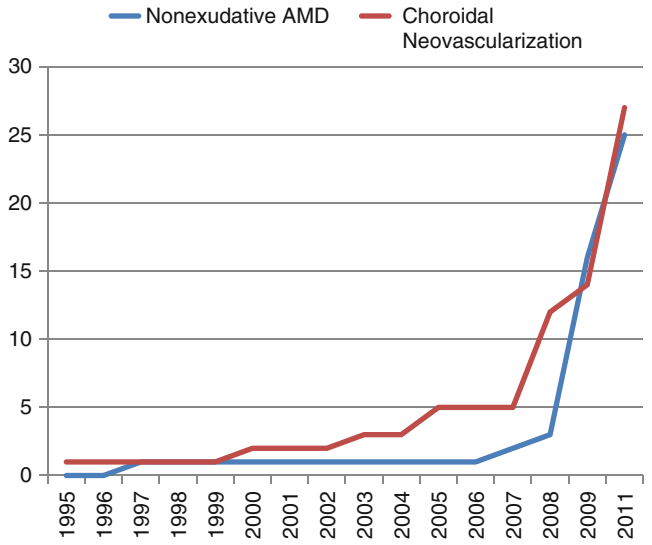

Fig. 1.1 The number of treatments for early- and late-stage AMD that are in clinical trials or are FDA-approved is increasing rapidly

of the complement pathway, (5) mitochondrial damage, (6) decreased RPE DICER 1 in eyes with GA, and (7) increased bone morphogenetic protein-4 in the RPE and extracellular matrix of eyes with drusen as well as those with GA.

1.2.1 Oxidative Damage

Epidemiological studies indicate that the strongest environmental risk factor for AMD is smoking [4]. Criteria for causal attribution in epidemiological studies include the following [5, 6]: (1) consistency of findings (between study types, settings, populations, and time), (2) strength of association, (3) evidence of dose–response (greater intensity and/or duration of exposure associated with greater effect), (4) evidence of reversibility (reduced risk with removal of exposure), (5) temporal relationship (evidence that exposure precedes effect), (6) biological plausibility (e.g., supporting biological evidence from animal and tissue models or other sources). All of these criteria have been fulfilled regarding the role of smoking and increased risk of AMD. Current smoking is associated with a 4.55-fold increased risk of neovascular AMD (vs. "never" smokers) and a 2.54-fold increased risk of atrophic AMD (vs. "never" smokers) [4]. Based on a pooled analysis of data, the risks of both neovascular and atrophic AMD seem to decrease once one stops smoking [4]. Some biological effects of smoking include decreased luteal pigments in the retina [7], decreased antioxidant levels [8], immune system activation [9], reduced choroidal blood flow [8], and potentiation of nicotine-stimulated angiogenesis [10]. Cigarette smoke contains inorganic and organic free radicals, including nitric oxide, peroxynitrite, and reactive oxygen species (e.g., superoxide) [11].

Mitchell et al. [12] found that smoking was associated with increased RPE abnormalities. In a murine model, RPE apoptosis and early features of AMD (e.g., loss of RPE basal infoldings, vacuole formation within the RPE, Bruch's membrane thickening) develop after chronic exposure to cigarette smoke [13]. In addition, cigarette smoke induces significant oxidative DNA damage in murine RPE [14]. With aging and early AMD, the retinal pigment epithelium (RPE) loses cuboidal morphology and becomes flattened or atrophic, particularly when overlying thickened Bruch's membrane [15]. Also, with aging and early AMD, the number of RPE cells seems to decline in the macula at a greater rate than in the periphery [16]. Apoptotic RPE cells and rod photoreceptors are present near the edge of GA [17]. These data indicate that RPE are a target of oxidative damage in AMD.

The Age-Related Eye Disease Study (AREDS, NCT00594672) showed that daily supplementation with zinc oxide, cupric oxide, beta-carotene, vitamin C, and vitamin E reduces the risk of moderate visual loss by approximately 19% during a 5-year period of follow-up [18]. The AREDS antioxidant supplements reduced oxidation of cysteine but had no effect on glutathione [19]. Thus, the beneficial effect of antioxidant supplementation on progression to advanced AMD may be partially explained by its effect on cystine and/or its effect on cysteine availability. Cysteine is an important antioxidant involved in regulation of apoptosis and immune function.

Zinc reduces reactive oxygen species by several mechanisms [20]. For example, it is an inhibitor of NADPH oxidase, is required for superoxide dismutase activity, and induces metallothionein. One interpretation of the AREDS results is that antioxidant supplementation reduces the risk of visual loss associated with AMD among properly selected patients, especially for patients with the *CFH*TT genotype [21].

Genetic studies also indicate that oxidative stress is important in the development of AMD. A mitochondrial DNA (mtDNA) polymorphism [22] and a superoxide dismutase polymorphism [23], for example, increase the risk for AMD. Also, a susceptibility locus near the hypothetical gene LOC387715 is associated with increased risk of AMD [24]. Some [25], but not all [26], evidence indicates that the latter locus codes for a mitochondrial protein.

Biochemical and histological studies have implicated oxidative damage as a possible cause of AMD. Eyes with GA exhibit DNA strand breaks and lipoperoxidation [27]. In some studies, RPE antioxidant enzyme changes (e.g., increased heme oxygenase-1 and -2) in AMD eyes indicate that the RPE cells are under oxidative stress [28]. Using microarray analysis of laser capture microdissected RPE, Ishibashi et al. [29] found that glutathione S-transferase M1 (GSTM1) was underexpressed in aging RPE. Whether due to genetic predisposition (the GSTM1 null genotype is seen in 50% of the white population) or due to aging, reduced GSTM1 activity might enhance RPE susceptibility to oxidative stress. (These studies did not confirm earlier studies by other groups in that they indicated there is not a significant *global* decline in antioxidant defenses in non-AMD aged RPE). Advanced glycation endproducts occur in soft drusen, in basal laminar and basal linear deposits, and in the cytoplasm of RPE cells associated with CNVs [30, 31]. Carboxymethyl lysine is present in drusen and CNVs [30, 32] as are carboxyethyl pyrrole protein adducts [30]. Additionally, Fe^{2+}, which is an essential element for enzymes involved in the phototransduction cascade, in outer segment disc membrane synthesis, and in the conversion of all-*trans*-retinyl ester to 11-*cis*-retinol in RPE, also catalyzes the conversion of hydrogen peroxide to hydroxyl radicals and is known to accumulate in Bruch's membrane in AMD eyes [33, 34].

Proteomic analysis of RPE from eyes with early AMD demonstrates decreased protein chaperones [35]. Proteins involved in protection from stress-induced protein unfolding and aggregation, mitochondrial trafficking and refolding, and regulating apoptosis (e.g., heat shock protein70 (HSP70) and α-A crystalline) change early in the disease process. Late-stage changes occur in proteins that regulate retinoic acid and regeneration of the rhodopsin chromophore. Decanini et al. [36] found that the content of several antioxidant enzymes and specific proteins that facilitate refolding or degradation of oxidatively damaged proteins increased significantly in late-stage dry AMD. These proteins are involved in the primary (copper–zinc superoxide dismutase, manganese superoxide dismutase, and catalase) and secondary (heat shock protein (HSP) 27, HSP 90, and proteasome) defense against oxidative damage. Additionally, the insulin prosurvival receptor exhibited disease-related upregulation. This pattern of protein changes in human donor tissue is consistent with the hypothesis that oxidative damage plays a role in the pathogenesis and progression of AMD [36].

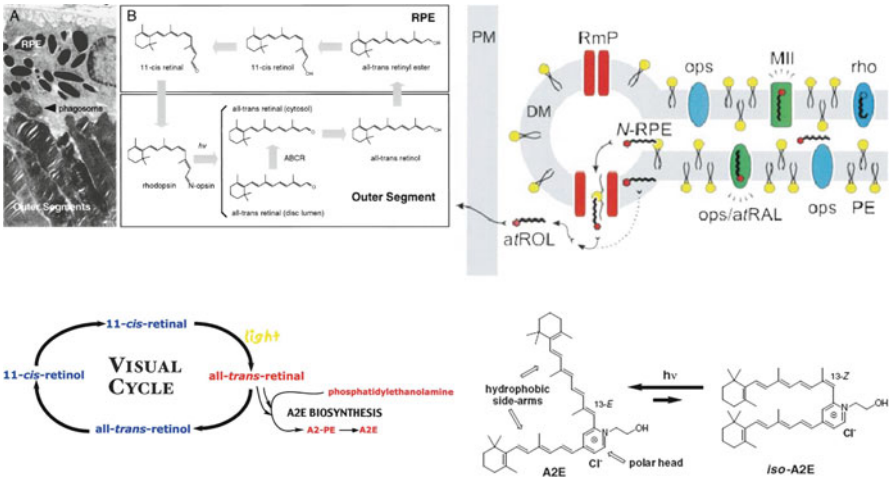

Fig. 1.2 The visual cycle and A2E formation. *Upper left*: The chemical reactions occurring in the photoreceptor outer segment and RPE are shown (ABCA4 is identified as ABCR in the figure) (reproduced with permission from Sun and Nathans [272]). *Upper right* ABCA4 (identified as Rim protein [RmP] in the figure) transports N-retinyledene-phosphatidylethanolamine (N-RPE) from outer segment discs to the photoreceptor cytoplasm. In the cytoplasm, all-*trans*-retinal (*at*RAL) is converted to all-*trans*-retinol (*at*ROL) (reproduced with permission from Weng et al. [40]). *Lower left*: In the outer segment discs, ethanolamine + 2(retinaldehyde) → N-retinylidene-N-retinylethanolamine (A2E) (reproduced with permission from Sparrow et al. [51]). *Lower right*: Structure of A2E (reproduced with permission from Sparrow et al. [51], see text for details)

1.2.2 Lipofuscin Accumulation

Excessive lipofuscin accumulation in the RPE may be important in AMD pathogenesis [37]. In RPE cells, the main source of lipofuscin probably is the undegradable components of phagocytized outer segments [38]. In vertebrate photoreceptors, light causes isomerization of the chromophore, 11-*cis*-retinyledene, to all-*trans*-retinyledene, followed by release of all-*trans*-retinal from the opsin binding pocket and its reduction to all-*trans*-retinol (Fig. 1.2) [39]. ABCA4, an ATP-binding cassette transporter present in the outer segment of rods and cones, transports N-retinyledene-phosphatidylethanolamine from the outer segment discs to the photoreceptor cytoplasm [40, 41]. Retinol dehydrogenase 8 (in outer segments) and retinal dehydrogenase 12 (in inner segments) reduces all-*trans*-retinal to all-*trans*-retinol [42, 43]. Vitamin A (all-*trans*-retinol) diffuses to RPE where it is esterified by lecithin/retinol acyltransferase to all-*trans*-retinyl esters and is stored in retinosomes [44, 45]. All-*trans*-retinyl esters are isomerized to 11-*cis*-retinol in a reaction involving RPE-65 [46–48]. Next, 11-*cis*-retinol is oxidized to 11-*cis*-retinal [49, 50] which then diffuses across the extracellular space to photoreceptors and recombines with opsin proteins to regenerate visual pigments. Within the outer segment discs, ethanolamine can combine with two retinaldehyde molecules to form N-retinylidene-N-retinylethanolamine (A2E); A2E is a major fluorophore in lipofuscin found in the RPE [51].

1.2.3 Chronic Inflammation

Several lines of evidence indicate that AMD is associated with chronic inflammation in the region of the RPE, Bruch's membrane, and the choroid [52]. Drusen, for example, contain many components of the activated complement cascade [53–55]. Anatomic studies demonstrate the presence of inflammatory cells in Bruch's membrane [56]. Bioactive fragments of C3 (C3a) and C5 (C5a) are present in drusen of AMD eyes and induce vascular endothelial growth factor (VEGF) expression in RPE cells [57]. The latter findings may explain why confluent soft drusen are a risk factor for CNVs in AMD eyes [57]. The presence of proinflammatory molecules in drusen constitutes a stimulus for chronic inflammation in the RPE–Bruch's membrane–choriocapillaris complex that may result in some features of late AMD. In addition to complement activation, retinal microglial activation and choroidal macrophage infiltration are part of the inflammatory response associated with AMD [58, 59].

One interpretation of the AREDS data is that zinc, one of the main therapeutic ingredients of this treatment, also affects the complement system, which in turn may slow disease progression. Zinc inhibits C3 convertase activity [60], and levels of C3a des Arg, which is an inactivated carboxypeptidase N cleavage product of C3a and reflects complement activation, are higher in patients with AMD vs. controls [61]. In addition, zinc is essential for normal functioning of cell-mediated immunity [20, 62, 63]. Apoptosis is potentiated by zinc deficiency, and zinc deficiency adversely affects the secretion and functions of various cytokines (e.g., interleukin [IL]-2, interferon [IFN]-γ) [64]. Zinc supplementation reduces intercellular adhesion molecule-1 (ICAM-1) levels and can reduce plasma markers of oxidative stress in humans. Also, zinc upregulates the zinc finger protein A20, which inhibits nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation.

Oxidative stress can augment inflammation. (The converse is also true.) Reactive oxygen species modulate the inflammatory system in part via enhanced expression of proinflammatory genes [65]. Nuclear factor erythroid-2 related factor 2 (Nrf2) is a basic leucine zipper transcription factor involved in cellular redox homeostasis (see Cano et al. [14] for references). The main function of Nrf2 signaling is to induce an antioxidant response (e.g., induce synthesis of NADPH:quinone reductase (NQO1) and glutamate-cysteine ligase regulatory unit [GCLM]) when cells are stressed oxidatively [66]. NF-κB and activator protein (AP)-1 are also induced by oxidative stress, but they do not regulate as comprehensive an antioxidant response [65]. Nrf2 is normally bound to Kelch-like ECH-associated protein 1 (Keap1) in the cytosol. When the multiple cysteine residues on Keap1 are exposed to reactive oxygen species, Keap1 undergoes a conformational change, and Nrf2 is released, translocates to the nucleus, dimerizes with transcription factor Maf proteins, and binds to the antioxidant response element (ARE), thus initiating DNA transcription of antioxidant response enzymes (e.g., heme oxygenase-1 and glutamate cysteine ligase, which generates glutathione and thioredoxin) [14].

Nrf2 signaling can modulate the innate immune system. Nrf2 signaling, for example, is known to modulate the immune response to sepsis in preclinical models [67]. Deficiency in detoxification and increased oxidative stress can foster the development of autoimmune disease (see discussion of parainflammation below). In a murine model of uveitis (intraperitoneal lipopolysaccharide), for example, Nrf2 signaling reduces the inflammatory response during acute intraocular inflammation [68], and in cutaneous wound healing models, deficient Nrf2 signaling predisposes to the development of chronic inflammation [69]. Nrf2 signaling also can modulate complement activation and C3 deposition in the brain [70]. In a murine model of chronic exposure to cigarette smoke, C3, C5, membrane attack complex, and to a lesser extent, CFH deposition in Bruch's membrane was present [71].

Recognition of smoking as a critical risk factor for developing AMD and identification of the molecular basis for smoking-induced alterations in retina–RPE homeostasis has led to the identification of potential strategies for treating AMD. For example, Nrf2 signaling regulates glutathione synthesis in RPE, and sulforaphane (1-isothiocyanotao(4R)-(methylsulfinil) butane), an Nrf2 activator, protects RPE cells from oxidative injury (induced by ultraviolet light) in model systems [72]. Oltipraz (4-methyl-5-pyrazinyl-3H-1,2-dithiole-3-thione)- and dimethylfumarate-induced upregulation of glutathione S-transferase and NADPH-quinone reductase, transcription of which is activated by Nrf2, reduces oxidation-induced RPE apoptosis in vitro [73, 74]. Zinc increases glutathione in RPE via a pathway regulated by Nrf2 [75]. In some systems, pharmacological induction of Nrf2 signaling can restore the age-related decline in Nrf2 transcriptional and nuclear translocation response [76]. Sulforaphane is present in broccoli sprouts and is an Nrf2 activator. Sulforaphane induces a conformational change in Keap1 that permits Nrf2 to translocate to the nucleus, bind to the ARE, and induce transcription [77]. Sulforaphane, oltipraz, and dimethylfumarate induce Nrf2 activation in RPE and photoreceptors [72–74, 78–80]. Triterpenoids, such as oleanolic acid, are steroid-like molecules with antioxidant and anti-inflammatory activity [77, 81], and this effect is mediated via Nrf2 signaling [82, 83]. (Triterpenoids form Michael adducts with cysteine residues on Keap1.) Triterpenoids are more potent activators of Nrf2 signaling than sulforaphane and were found to be safe in a phase 1 trial of patients with solid tumors [83]. The triterpenoid, 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole (CDDO-Im), reduced inflammation and induced antioxidant enzyme expression in a murine model of uveitis [68]. CDDO-Im also maintains normal RPE basal infoldings, prevents cytoplasmic vacuole formation, and maintains normal Bruch's membrane thickness in mice exposed to cigarette smoke for 6 months [14].

Inflammation caused by stressed cells (e.g., free radical injury) may be of lower magnitude than that caused by more noxious events (e.g., infection) and has been termed parainflammation [84]. Parainflammation in the aging retina has been reviewed in detail by Xu et al. [85]. When insults such as mechanical trauma damage tissue, repair can be initiated by the innate immune system via a mechanism called "recognition of reduced or altered self" [86–88]. Autoantigens that can bind to receptors on antigen-presenting cells (e.g., interphotoreceptor retinoid binding

protein) induce chemotaxis that may be involved in the promotion of tissue repair [87]. These potent immunostimulants (e.g., defensins, cathelicidin, eosinophil-derived neurotoxin, and high-mobility group box protein 1) serve as early warning signals to activate innate and adaptive immune systems and have been termed, Alarmins [88]. Alarmins are released rapidly in response to infection or tissue injury, recruit and activate antigen presenting cells (e.g., dendritic cells and macrophages), and exhibit potent immunoenhancing activity [88].

Although the physiological outcome of parainflammation is restoration of tissue functionality, sustained tissue malfunction results in a chronic parainflammatory state and can be associated with the development disease progression [85]. Examples of nonocular conditions associated with chronic low-grade inflammation include obesity and allergy (exhibiting maladaptive host responses to noxious conditions resulting from a shift in homeostatic set points) and type 2 diabetes, Parkinson disease, and Alzheimer disease (nonadaptive conditions resulting from dysregulation of parainflammation) [84, 85, 89, 90]. Xu et al. [85] have pointed out that parainflammation in the vasculature may contribute to parainflammation in age-related diseases and may be the initiating event in these diseases [91].

As noted above, reactive oxygen (O_2^-, H_2O_2, OH^-) and nitrogen species (e.g., NO_3^-, NO_2^-, $ONOO^-$) are generated as a result of normal physiological events in the retina such as outer segment phagocytosis by RPE cells. Oxidative and nitrative stress can trigger parainflammation [85]. Xu et al. [85] have posited that oxidized low-density lipoproteins, resulting from oxidative damage to outer segment unsaturated lipoproteins, may play a particularly important role in promoting parainflammatory responses in the aging retina by binding to retinal microglia and RPE cells via scavenger receptors (e.g., CD36, CD68, LOX-1 [92]). Normally complement factor B is expressed in the apical portion of the RPE cells, but with age, complement factor B expression increases and extends to the basal portion of the RPE [93]. Upregulation of factor B is associated with C3 and C3a deposition in Bruch's membrane and the basal RPE.

Thus, with age [93] and with oxidative stress [94–96], RPE cells can produce inflammatory cytokines and chemokines that initiate or contribute to parainflammation. There is increased leukocyte infiltration of the aging choroid, which may mean that parainflammation is part of normal choroidal aging [85]. At this time, it seems reasonable to speculate that the inflammatory processes that contribute to AMD progression are closely related to the parainflammatory response described above.

1.2.4 Complement Mutations

Drusen, GA, and CNVs are associated with mutations in components of the complement pathways, which is part of the innate immune system (Fig. 1.3). Protective and risk-enhancing mutations in components of the complement pathways have been reported and include the following loci: complement component 1 (C1), complement component 2 (C2), complement factor B (CFB), complement component 3 (C3), complement component 7 (C7), complement component 9 (C9), factor B

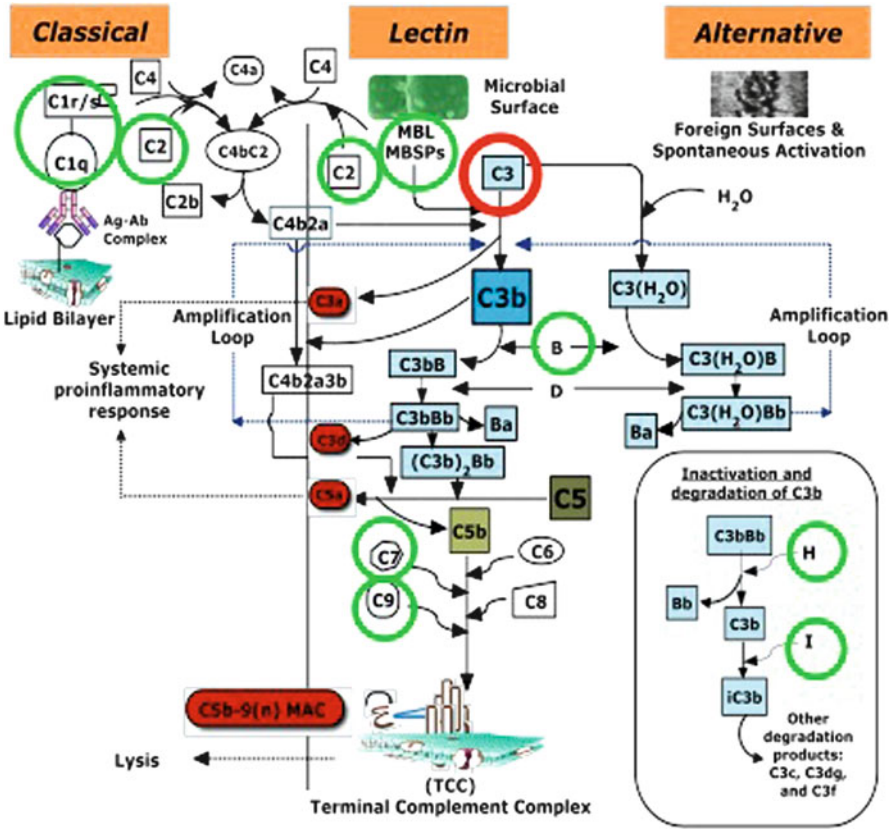

Fig. 1.3 AMD-associated mutations in the complement cascade. Four major activation pathways for the complement system are known, and three of these are illustrated. (The fibrinolytic-activated *intrinsic* pathway is not shown.) Activation of the complement system plays an important role in immunity. Inappropriate complement activation can damage tissue. Multiple complement components have been linked to AMD (*green circles*), including drusen, GA, and CNVs. Complement C3 (*red circle*) is the key point of convergence of all activation pathways

(CFB), complement factor H (CFH), factor I (CFI), and complement factor H-related 1 and 3 (CFHR1/CFHR3) [53, 97–111]. Regarding the protective CFH haplotype tagged, in part, by a deletion of two members of the CFH gene family (CFHR1 and CFHR3), in two cohorts, deletion homozygotes represented 4.9 and 6.7% of controls vs. 1.2 and 0.94% of cases, respectively [97]. Anderson et al. [98] have pointed out that CFHR1 and CFHR3 possess significant amino acid sequence homology and also share binding properties with CFH. In contrast with CFH, which regulates the C3 convertase, however, CFHR1 appears to act downstream by modulating the activity of the C5 convertase and inhibiting formation of the membrane attack complex. Thus, the protective effect conferred by deletion of CFHR1/CFHR3 in AMD may be mediated by removal of the C5a blockade and disinhibition of membrane attack complex formation [112].

Oxidative damage can compromise regulation of the complement system by the RPE. Thurman and Holers [113] noted that the alternative complement pathway is continuously activated in the fluid phase, and tissue surfaces require continuous complement inhibition to prevent spontaneous autologous cell injury. Sohn et al. [114] demonstrated that the complement system is continuously activated in the eye. Thurman et al. [115] showed that oxidative stress reduces the regulation of complement on the surface of ARPE-19 cells by reducing surface expression of the complement inhibitors CD55 and CD59 and by impairing complement regulation at the cell surface by CFH. Sublytic activation of the complement cascade also causes VEGF release from the cells, which compromises RPE barrier function. Similarly, oxidative stress can reduce the ability of IFN-γ to increase CFH expression in RPE cells [116]. In vitro evidence indicates that products of the photo-oxidation of A2E in RPE cells can serve as a trigger for the complement system [117]. Thus, the relative abundance of lipofuscin in submacular RPE may predispose the macula to chronic inflammation and AMD, particularly in patients who cannot control complement activation due to inherited abnormalities in the complement system. Hollyfield et al. have described an animal model that links oxidative damage and complement activation to AMD [118]. Some AMD-risk enhancing mutations not directly involving the complement pathway also are linked to inflammation or oxidative damage [25, 119–125].

1.2.5 Mitochondrial Damage

With increasing age, there is increased mtDNA damage and decreased DNA repair enzyme capability in rodent RPE and choroid. Wang et al. [126] demonstrated that drusen in AMD donor eyes contain markers for autophagy and exosomes. Using in vitro modeling of increased mtDNA damage induced by rotenone, an inhibitor of mitochondrial complex I, in the RPE, Wang et al. [126] found that exosomes released by the stressed RPE are coated with complement and can bind CFH. Thus, increased autophagy and the release of intracellular proteins via exosomes by aged RPE may contribute to drusen formation.

RPE abnormalities and atrophy, resembling clinical findings in AMD, are present in the majority of individuals with the mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes syndrome (MELAS) (associated with the A3243G mtDNA mutation), which may indicate that mitochondrial abnormalities play a role in AMD pathogenesis [127]. Furthermore, Jones et al. [123] found that mtDNA haplogroup H was associated with a reduced prevalence of any (early and late) age-related maculopathy. Haplogroup J was associated with a higher prevalence of large, soft distinct drusen, and haplogroup U was associated with an increased prevalence of RPE abnormalities. AMD is associated with decreased mitochondrial number and area and a loss of cristae and matrix density [128], increased RPE mtDNA damage, and decreased repair, as well as decreased mitochondrial respiration [129].

Jarrett et al. [129] identified several factors that place mitochondria at high risk for oxidative damage. First, mitochondria represent the major source of endogenous reactive oxygen species in most cells. Second, phagocytosis of outer segments, which produces reactive oxygen species [130], has been shown to damage mtDNA [131]. Third, mtDNA, which may be the most important target of oxidative damage within mitochondria, is especially susceptible to oxidative damage [132]. Features of mtDNA that create this risk include the following: (1) lack of protective histone and nonhistone proteins and mtDNA's association with the inner mitochondrial membrane (the site of electrophile and oxidant production as well as transition metals, which can promote Fenton chemistry that generates $OH^{\bullet}$) [133]; (2) mtDNA is intron-less, which means that DNA damage is certain to affect a portion of the genome that codes for enzymes involved in oxidative phosphorylation or DNA translation; (3) mtDNA has a high transcription rate, which increases the probability of mutations and/or deletions; and (4) the mtDNA repair mechanism may not be adequately efficient [134]. The consequence of RPE mitochondrial damage is reduced metabolic activity (e.g., reduced efficiency of outer segment phagocytosis) [135] and/or apoptosis. (Cytochrome c release from the mitochondria to the cytosol, where it binds apoptotic peptidase activating factor 1, is the initiating event for the internal apoptotic pathway).

Reactive oxygen species that may be relevant to AMD pathobiology include H_2O_2 superoxide (O_2^-), singlet oxygen ($^1O_2$), hydroxyl radical ($OH^{\bullet}$), nitric oxide (NO), peroxinitrite ($ONOO^-$), and lipid peroxyl radicals. Superoxide and hydroxyl radical are produced within the mitochondria [129]. Mitochondria are a major source of intracellular reactive oxygen species, as normal mitochondrial metabolism generates superoxide, which can foster the production of hydroxyl radical and peroxynitrite and damage to aconitase [136–138]. Oxidative damage to aconitase can result in the release of Fe^{2+} and H_2O_2 as well as disruption of the tricarboxylic acid cycle. As noted above, other chromophores within RPE, such as lipofuscin and retinoid metabolites (e.g., A2E), also can initiate oxidative damage.

Recognition of the role of mitochondrial damage in AMD progression may lead to new therapeutic strategies involving the use of agents that target mitochondria [129]. MitoQ (a triphenyl-phosphonium cation-linked derivative), for example, is mitotropic and can be used to target antioxidants to mitochondria [139, 140]. MitoQ is in clinical trials for treatment of Parkinson disease [141]. Nanotechnology approaches have led to the development of novel mitotropic agents such as Bolasomes, mitochondrial targeted liposomes, and mitochondrial targeted nanoparticles [142]. These molecules can target antioxidants to mitochondria.

1.2.6 DICER 1

Dicer enzymes cut long double-stranded RNA molecules into shorter pieces, and play an important role in gene-silencing pathways that involve short interfering RNA or microRNA (miRNA) [143]. Kaneko et al. [144] showed that the

miRNA-processing enzyme, DICER1, is reduced in the RPE of AMD eyes with GA, and that conditional ablation of DICER1, but not seven other miRNA-processing enzymes, induces RPE degeneration in mice. DICER1 knockdown induces accumulation of *Alu* RNA in human RPE cells. (*Alu* RNAs are transcripts of *Alu* elements, which are the most common noncoding, repetitive DNA sequences in the human genome named for the restriction site common to all Alu elements: the target site for the restriction endonuclease Alu I, obtained from the bacteria *Arthrobacter luteus*.) Kaneko et al. [144] also demonstrated that *Alu* RNA is increased in the RPE of AMD eyes with GA, and this pathogenic RNA induces human RPE cytotoxicity and RPE degeneration in mice. Furthermore, antisense oligonucleotides targeting *Alu* RNAs prevented DICER1 depletion-induced RPE degeneration despite global miRNA downregulation. DICER1 degrades *Alu* RNA into shorter sequences that presumably are nontoxic, and this digested *Alu* RNA cannot induce RPE degeneration in mice. These findings reveal a miRNA-independent cell survival function for DICER1 involving retrotransposon transcript degradation, show that *Alu* RNA can directly cause human pathology, and identify new targets for treating GA.

DICER1 is downregulated in chemically stressed cells, but DICER1 is not reduced in the RPE of human eyes with vitelliform dystrophy, retinitis pigmentosa (RP), or retinal detachment [144]. Caspase-3 cleavage was observed in the RPE cells of BEST1 Cre; Dicer1f/f (flox) mice and in *Alu* RNA-stimulated or -overexpressing human RPE cells. These data indicate a role for *Alu* RNA-induced RPE cell apoptosis triggered by DICER1 dysregulation in GA. The inciting events that trigger an RPE-specific reduction of DICER1 in patients with GA are unknown. Of note, however, Kaneko et al. [144] found that oxidative stress may play a role, as they demonstrated that hydrogen peroxide downregulates DICER1 in human RPE cells.

1.2.7 Bone Morphogenetic Protein-4

Bone morphogenetic protein (BMP)-4 is an important regulator of differentiation, senescence, and apoptosis in many different cells and tissues. BMP-4 is involved, for example, in chemotherapy-induced senescence of lung and prostate cancer cells. BMP-4 acts as a mediator in oxidative stress-induced senescence. Via Smad and the p38 signaling pathway, BMP-4 increases and activates p53 and $p21^{Cip1/WAF1}$ and decreases phospho-Rb. BMP-4 is highly expressed in the RPE and adjacent extracellular matrix of patients with dry AMD [145]. In vitro studies show that sublethal oxidative stress increases BMP-4 expression in RPE, and both BMP-4 and persistent mild oxidative stress can induce RPE senescence through the p53-$p21^{Cip1/WAF1}$-Rb pathway [145]. Oxidative stress-induced senescence can be blocked by Chordin-like, an antagonist of BMP-4, or SB203580, a phospho-p38 inhibitor [145].

Transforming growth factor (TGF)-β is involved in mediating oxidative stress-induced premature senescence of fibroblasts. TGF-β mediates oxidative stress-induced RPE cell senescence through the up-regulation of $p21^{WAF1/cip1}$ and the down-regulation of phosphorylated Rb, and blockade of TGF-β signaling by specific TGF-β

antibody can impede RPE senescence [146]. TGF-β and BMP-4 may have a synergistic effect in mediating the oxidative stress-induced RPE senescence because neither TGF-β antibodies nor BMP-4 antagonist alone can completely block the expression of senescence marker genes to baseline in the oxidative stress-treated RPE cells [145].

Zhu et al. [147] reported that RPE cells induced into senescence by chronic oxidative stress secrete fourfold higher IL-8 than nonsenescent RPE cells. IL-8 promotes angiogenesis by increasing the proliferation, survival, and migration of endothelial cells and promotes inflammation by increasing neutrophil chemotaxis and degranulation. Senescent heterogeneity combined with the effects of other cytokines (e.g., TNF-α inhibition of BMP-4 expression) may drive some cells to senescence with GA and others to CNV stimulation [147].

A proposed pathogenesis (Fig. 1.4) of AMD suggests the possibility of therapeutic intervention at different points in the natural history of the disease with antioxidants, visual cycle inhibitors, anti-inflammatory agents, antiangiogenic agents, and neuroprotective agents.

1.3 Treatment

Various pathway-based therapies for AMD have been reviewed extensively elsewhere [148]. Here we update some of this information.

1.3.1 Antioxidants

The AREDS did not show a statistically significant benefit of the AREDS formulation for either the development of new GA or for involvement of the fovea in eyes with preexisting atrophy [18]. In part, this result may be due to the paucity of GA patients in the study. Carotenoids and omega-3 (ω-3) fatty acids were not studied in the AREDS. Carotenoids (e.g., lutein, zeaxanthin) have potentially therapeutic biological effects: filter blue light (high energy) [149]; antioxidant (scavenge singlet oxygen, quench triplet state of photosensitizers, retard peroxidation of membrane phospholipids) [150, 151]; and reduce chromatic aberration [149]. They are derived from diet [152], are transported in serum on circulating lipoproteins [153], and are concentrated in the macula [154]. Some [155, 156], but not all [157], studies indicate that higher dietary intake of lutein and zeaxanthin reduce the risk of AMD. In addition, some studies indicate that dietary β-carotene *increases* the risk of AMD [155]. Omega-3 fatty acids are essential and are derived from diet in humans; ω-3 fatty acids include α-linoleic acid (short-chain, precursor to docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)), EPA (long-chain, precursor to DHA, antithrombotic, hypolipidemic), and DHA (long-chain, main lipid constituent of outer segment membranes) [158]. A meta-analysis of 9 studies (3 prospective cohort,