Oxidative stress: The achilles' heel of neurodegenerative diseases of the retina

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1. ABSTRACT

Age-related macular degeneration (AMD) is the leading cause of blindness among adults in the developed countries. It is characterized by the progressive loss of central vision. AMD is classified into two forms: dry and wet. Dry AMD involves the accumulation of deposits in the RPE and Bruch’s membrane; Wet AMD is characterized by neovascularization in the choroid. Whether the two forms of AMD share the same mechanism for the disease development is presently not clear. Oxidative stress, inflammation, and ER-stress are the common modes for the pathogenesis of AMD. In addition, other risk factors and several signaling pathways have been implicated as causative factors of AMD. In this paper, the mechanisms underlying AMD, risk factors involved in the pathology, representative animal models, and therapeutic treatment strategies are reviewed.

2. INTRODUCTION

AMD, a severe ocular disease, is among the most prevalent of ocular diseases. It occurs in adults at an age above 50 (1-2) with the loss of central vision and visual acuity. AMD development is progressive. By means of genetics, pathology, physiology, anatomy, biochemistry, animal models and functional research, a better understanding of the progression of AMD has been achieved. However, information about the mechanism underlying AMD is still insufficient, contributing to the absence of an effective treatment for AMD. This review paper is based on recent publications and identifies risk factors which contribute to the etiology as well as possible pathways involved in the disease development and therapeutic strategies for the treatment of AMD.
3. CHARACTERISTICS OF MACULAR DEGENERATION

Macular degeneration, an alternative name for age-related macular degeneration (AMD), is a heterogeneous human eye disorder that damages the center of the retina (called macula); it is the leading cause of irreversible blindness among the adults over the age of 50 years; and is one of the major blinding diseases in the world (1-2). AMD is characterized by progressive loss of central, high-acuity vision due to photoreceptor degeneration in the central retina. AMD may exhibit the variable presence of soft and hard drusen in Bruch’s membrane (BM) between the retinal pigment epithelium (RPE) and the choroid with or without evidence of damage to the underlying RPE, deposition of lipofuscin in the RPE, hyperpigmentation and hypopigmentation of the RPE. These features may occur in association with choroidal neovascularization (CNV), atrophy of RPE, geographic atrophy, neurosensory detachment, and fibrous scarring (3-4). The development of AMD is a progressive process and clinically it can be subdivided into early and late stages (2-3, 5). Early stage AMD exhibits normal visual acuity, but moderate vision loss associated with the focal lesion (drusen) or diffuse lesion (basal deposits) in BM in the fundus (2, 5). Late stage AMD involves CNV formation, in which blood vessels of the choriocapillaris grow into or through the BM; RPE detachment, which results in fluid accumulation between the RPE and BM; thickened BM with drusen; a high level of deposits of autofluorescence lipofuscin in RPE cells and loss of photoreceptors (2). Clinically, late stage AMD usually can be classified into two forms: “dry” and “wet” AMD (6). Dry AMD (geographic atrophy) counts for 80% of AMD cases and is characterized by focal degeneration of photoreceptors, RPE and choriocapillaris in the macula and impairs visual acuity over time, while wet AMD (neovascular) counts for 20% of the cases, and results in sudden and acute vision loss in patients due to choroidal neovessel development (6).

Currently it is not clear whether the disease processes and the genetic and molecular pathways that lead to the different phenotypes of both forms of AMD are related or distinct. Studies revealed that bone morphogenetic protein-4 (BMP4) is differentially expressed in dry and wet AMD. In dry AMD, BMP4 is highly expressed in RPE and mediates oxidative stress induced RPE senescence in vitro via Smad and P38 pathways whereas in wet AMD lesions, BMP4 expression in RPE is low. Evidence suggests that BMP4 is regulated by tumor necrosis factor (TNF) and may be the molecular switch determining which phenotypic pathway is taken in the progression of AMD (7-8). Furthermore, proteomic analysis of a total of 901 proteins from different developmental stages (early/mid stage or advanced stage) of dry or wet AMD and normal macular regions indicated that 56 proteins were elevated and 43 were reduced in AMD patient tissues compared to normal controls. About 60% of the elevated proteins are involved in immune responses and host defenses, including many complement proteins and damage-associated molecular pattern proteins.

These results indicate that different pathways are involved in the progression of the dry and wet AMD (9).

4. MECHANISM OF AMD

AMD has a complex and multi-factorial etiology. Genetic factors, oxidative stress, inflammation, age, caucasian race (ethnicity), high-fat diet, iron, color of the iris, obesity, hypertension, hypercholesterolemia, etc. are all contributors to the pathogenesis of AMD (10). Several pathogenic pathways have been proposed including oxidative stress-induced damage and apoptosis (11), RPE cell dysfunction with accumulation of lipofuscin and impairment of lysosomal functions (12) as well as inflammatory processes with complement activation (13).

4.1. Heredity effect (genetic variations)

The variation within the genome may occur at a single base pair (single nucleotide polymorphisms, SNP) or copy number variation of small blocks of sequences (14).

4.1.1. Complement system

The complement system is composed of more than 30 components and regulators. As an innate immune defense mechanism, the complement system has been suggested to play a significant role in the pathogenesis of AMD [see review (15)]. Complement activation involves three principal pathways which converge at C3 (complement component 3) activation and results in the generation of several effectors and ultimately the terminal MAC (membrane attack complex) which is a complex of C5b, C6, C7, C8, and C9, and which can directly cause lysis of the cells through the formation of lytic pores in the cell membrane. Several regulatory proteins, such as factor H (CFH), decay accelerating factor (CD55), membrane cofactor protein (CD46), and membrane inhibitor of reactive lysis (MIRLC or protectin (CD59), control the complement cascade and protect autologous cells from complement attack [see review (15)]. CFH is secreted by RPE and is present in drusen (16). It has been proposed as an inhibitor of C3 convertase and other complement pathways [see review (14-15)]. Its polymorphism, tyrosine-histidine substitution at amino acid 402 (Y402H polymorphism), could be a risk allele associated with AMD (17). FOXO3 (forkhead box, type O, member 3) belongs to the FOXO family of forkhead transcription factors which are key downstream targets of the phosphatidylinositol 3-kinase-Akt pathway. Analysis of the sequence and activity of mouse CFH promoter demonstrated that the FOXO3 acetylation mediated oxidative stress-induced CFH suppression, e.g., under oxidative stress conditions, FOXO3 preferentially binds to the CFH promoter, causing induction of CFH expression (18). On the other hand, selective knockdown of ocular CFH in a laser-induced mouse CNV model resulted in increased MAC deposition and lead to the early onset and exacerbation of CNV (19). However, other reports demonstrated that polymorphisms of the CFH gene which are in the noncoding regions (LOC 387715) are strongly associated with AMD and suggested it modulates the risk of AMD not by disrupting protein function but rather by regulating the CFH gene expression (20). Recently other complement-regulatory proteins such as...
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complement factors C2, C3, C5 and B have been reported to be risk factors for developing AMD (4, 15).

4.1.2. Other genes and DNA variations

Several other genes and DNA variations have been suggested to be associated with AMD. The DNA repair enzyme gene, XPD (xeroderma pigmentosum complementation group D) at codon 751 Gln/Gln genotype may have a protective effect against development of AMD as its presence is significantly lower in the dry AMD patients (21). Manganese superoxide dismutase (MnSOD) gene polymorphism (Ala-9Val sequence polymorphism) is more much frequently detected in both types of AMD patients (22).

Apolipoprotein E (ApoE) deficient mice at ages of 6, 12 and 20 weeks old showed a decrease of antioxidant enzymes, an increase of lipofuscin accumulation and pro-inflammatory cytokine expression (23). A combination of three established AMD risk factors, aging, high fat cholesterol-rich (HF-C) diet, and APOE genotype, was demonstrated, using human apoE-targeted replacement (TR) or knockin mice, to be sufficient to induce AMD (5). In the aging apoE4 TR mice fed a HF-C diet, extensive degenerative alterations were seen at 65-127 weeks old of age in the retina/RPE/choroid. These changes are comparable to the pathology of human AMD including RPE hyperpigmentation, hypopigmentation, atrophy, BM thickening, soft drusenoid deposits, retinal neovascularization (RNV) and choroidal neovascularization (CNV) (5). Beta-amyloid (Abeta) was immunolocalized within the neovascularization (NV) and sub-RPE deposits in the aged apoE4 TR mice fed a HF-C diet, and within drusen and CNV in human AMD suggesting that apoE may promote amyloid deposition in AMD in an APOE4 allele-dependent manner (5).

4.2. Oxidative stress, inflammation and ER stress

Reactive oxygen species (ROS) include free radicals (O_2^·, OH·, H_2O_2, ROO·), hydrogen peroxide (H_2O_2), or singlet oxygen (1O_2). They may be produced as byproducts of cellular metabolism, or as byproducts of enzymatic reactions (24). Production of ROS and chronic oxidative stress lead to modification and damage of carbohydrates, membrane lipids, proteins and nucleic acids, which initiate the pathogenesis of many diseases [see review (25)]. The retina is the most frequently damaged organ following light exposure and has the highest rate of ER stress can be caused by many stimuli including oxidative stress, inflammation and other stress.

Chronic inflammation in the RPE cells has been implicated as a causative factor in AMD (39). Inflammatory cytokines IL-1 (interleukin-1), IFN (interferon), TNF, and various growth factors such as PDGF (platelet-derived growth factor) and FGF (fibroblast growth factor) have been shown to be involved in the pathogenesis of AMD (40). In Aberc mice, A2E (N-retinylidene-N-retinylethanolamine) accumulation causes enhanced secretion of VEGF (vascular endothelial growth factor), higher levels of expression of oxidative stress genes, complement activation, down regulation of complement-regulatory proteins and chronic inflammation of the RPE (41).

The endoplasmic reticulum (ER) is an important cellular organelle in which proteins are synthesized, folded, sorted and Ca^{2+} is stored. In recent years, more and more evidence implicating ER stress (protein folding stress), protein misfolding and aggregation as primary causes for many neurodegenerative diseases (42-44) has been accumulated. ER stress can be caused by many stimuli including oxidative stress, inflammation and other stress.
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conditions (42). It can be recognized by three transmembrane proteins called IRE1 (inositol-requiring protein-1), PERK (protein kinase RNA-like ER kinase) and ATF6 (activating transcription factor-6) which act as ER-resident sensors. In response to ER stress the cells generate a self-protective UPR (unfolded protein response) signaling cascade through protein kinases and transcription factors by regulation of ER chaperons, antioxidants and other guardian molecules (42). Under ER stress, the UPR signaling pathways are initiated for the protein proper folding and protein degradation to prevent cellular damage. If the ER stress is prolonged, apoptosis will eventually be initiated by regulation of caspases and CHOP (CCAAT/enhancer-binding protein homologous protein) expression (42-44). Proteomic analysis of retinas from human AMD patients demonstrated that chaperon proteins HSP60 (heat shock protein) and HOP (Hsp70/Hsp90 organizing protein) which are responsible for protein folding, were down regulated and a group of proteins involved in microtubule formation and regulation were also decreased in expression levels (45). The precursor to ER resident chaperon protein ERp29, which is associated with neurodegenerative diseases, was significantly lower in the eye of Ccr2−/−/Cx3cr1−/− [chemokine CCL receptor (CCR2) and chemokine C-X3-C receptor 1(CX3CR1) double knockout] mice (46). These down regulated proteins may result in the blockage of protein trafficking, and subsequent accumulation of unfolded protein aggregates and deposition of drusen and lipofuscin which eventually cause damage to RPE and BM.

Many other genes and factors are shown to be involved in oxidative stress. Tissue factor (TF), the primary initiator of blood coagulation initiates intracellular signaling and promotes inflammation and angiogenesis. TF was shown to be up regulated in the retinas of AMD patients and Ccl2−/−/Cx3cr1−/− double knockout mice. LPS (lipopolysaccharide) and H2O2 treatment of ARPE-19 cells increased TF expression (47). PPARs (peroxisome proliferator-activated receptors) belong to the steroid/thyroid nuclear receptor superfamily of ligand-activated transcription factors and play a role in AMD progression by regulating VEGF, MMPs (matrix metalloproteases), DHA, heme-oxgenase-1 (HO-1) and ROS (48). PPAR gamma, one of the three subtypes of PPARs, has been reported to be expressed in ocular tissue, specifically in RPE cells (49). In Ccl2−/−/Cx3cr1−/− mice, AMD patients and H2O2 treated ARPE-19 cells, the expression of PPAR gamma and its downstream proteins, VEGF, MMP-9 and HO-1 was increased (49). IL-6 (interleukin-6), a key factor in the modulation of immune responses and inflammatory processes was increased in AMD whereas its production was stimulated by H2O2 in ARPE-19 cells (50)

Knockdown of superoxide dismutase 2 (SOD2) in mitochondria by ribozyme (AAV-Rz432) in C57BL/6J mice resulted in decreased levels of MnSOD expression, elevated levels of markers of oxidative damage (nitrated and carboxyethylpyrrole-modified proteins) in the RPE-choroid. The mice exhibit multiple phenotypes similar to human AMD such as thickened BM, degeneration of RPE, shortened and disorganized photoreceptor outer and inner segments, apoptotic cell death, increased autofluorescence and elevated levels of A2E and iso-A2E (51).

Oxysterols are oxidation products of cholesterol that result from either autoxidation or enzymatic oxidation. Abnormal oxysterol levels can cause oxidative stress, inflammation and apoptotic cell death (52). The oxysterol pathway has also been proposed as a unifying hypothesis for the cause of AMD (52). ARPE-19 cells treated with cholesterol oxidation metabolite 27-hydroxycholesterol (27-HOC) for 24 hours increased levels of Abeta peptide production, ER stress markers, and oxidative stress-activated protein nucleus factor-kappaB (NF-kB) and HO-1. Furthermore, 27-HOC causes depletion of ER Ca2+ stores and glutathione, ROS generation, inflammation and apoptosis-mediated cell death (53).

Ubiquitin proteolytic system (UPS), distributed in different retinal cell types, had been demonstrated in the protection of retina from oxidative stress by mediating the degradation of misfolded and oxidative-damaged proteins without selection through modulation of the stability and activity of transcription factor Nrf2 (nuclear factor erythroid-derived 2, like 2) (54-55). However, the UPS only degraded the newly synthesized proteins (55-56). The accumulation of protein aggregates in AMD patients are newly-synthesized oxidative-damaged proteins. So it is possible that enhancing the capacity of UPS to degrade these proteins may provide a new strategy for treatment of AMD.

Several signaling pathways have been proposed to correlate with AMD including VEGF-A/VEGF-R2/Pi3K/Akt (57), PERK/eIF2a/ATF4 and IRE1/ASK1/JNK cascade (42). A missense mutation in fibulin-3 protein (R345W) caused protein accumulation in the ER of RPE cells which triggered the activation of UPR and lead to the increase of VEGF expression. This evidence suggests a possible link between ER stress and wet AMD (58).

4.3. Cigarette smoking

Cigarette smoke is the most important environmental risk factor contributing to both dry and wet AMD pathogenesis (59) by causing oxidative damage and RPE cell death (60). ARPE-19 cells and primary human RPE cells exposed to cigarette smoke extract or a component of cigarette smoke (hydroquinone, HQ) showed oxidative damage, reduced cell viability and apoptosis including reduction in cell size and nuclear condensation, increased lipid peroxidation (determined by increased synthesis of 4-hydroxy-2-nonenal, 4-HNE) and mitochondrial superoxide production, and decreased intracellular glutathione (GSH) which is an important antioxidant that aids in eliminating toxic chemicals (61). However, cigarette smoke also induced the expression of VEGF, HO-1 and the transcription factor Nr2f2 (61). Two month old C57BL/6J mice exposed to smoke for 5-hour/day, 5-day/week for 6 months produced oxidative stress-associated features compared to control mice, including an increase in 8-oxo-7,8-dihydro-2'-
PEDF (pigment epithelium-derived factor) protein was induced oxidative injury. VEGF protein was increased and detected in RPE from C57BL/6J mice of HQ-treated mice (67). Furthermore, heat shock protein 27 (Hsp27), a key regulator of actin filament dynamics, is upregulated in RPE from patients with AMD, and Hsp25, p38, and ERK (extracellular signal-regulated kinase) phosphorylation are increased in aging C57BL/6J mice which were chronically exposed to HQ, suggesting that phosphorylated Hsp27 might be a key mediator in the pathogenesis of AMD along with actin reorganization and bleb formation (64).

4.4. Other factors for AMD

AMD patient retinas contained more iron than the healthy subjects which suggests that excessive iron can cause damage to protein, lipids and DNA through the generation of free radicals in the Fenton reaction (65). Ceruoplasmin (Cp) and hephestin (Heph) are multicopper ferroxidases and can facilitate iron export from the cells. Iron can be taken up by the serum transport protein transferrin (66). In a Cp and Heph double knockout mouse model which exhibits both wet and dry AMD characteristics, iron overload was found in the retina in an age-dependent manner with a reduced level of transferrin receptor (66).

Recently, Lyzogubov et al 2011 subretinally injected 1 mg polyethylene glycol-8 (PEG-8) to wild type mice and caused elevated levels of C3 split products, C9, TGF (transforming growth factor) -beta 2, bFGF (basic fibroblast growth factor) at post injection day 1, and the progression of developed CNV to penetrate into the BM at day 3, fully developed CNV and retinal degeneration occurred at post injection day 5 (67).

5. ANIMAL MODELS OF AMD

Mice have been widely used to generate models for investigation of the pathogenesis of human AMD. Because of the complication in the pathology of AMD, there is no good mouse model displaying all the phenotypes of AMD. For example, the mouse has no macula, a lower ratio of cone to rod, drusen is rarely seen due to their simpler BM and different lipofuscin extrusion compared with humans. However, focal atrophy of photoreceptor and RPE, lipofuscin accumulation, and increased A2E levels can develop in aged mouse eyes, which are representative of the distinct characteristics of AMD (68). AMD models can be generally divided into three groups (68). In the first group are genetically engineered mice that target genes related to juvenile macular dystrophies including Abcr knockout (recessive Stargardt disease) and transgenic ELOVL4 (dominant Stargardt disease) or target inflammatory genes relevant to AMD such as Ccl2, Cfh-/-, Cx3cr1-/-, and Ccl2-/-/Cx3cr1-/-. Also, oxidative stress associated genes such as Sod1 and Sod2 knockdown and metabolic pathway genes such as transgenic mcd/mcd (cathepsin D), and transgenic ApoE4 on high fat and high cholesterol diet (lipid metabolism) are in this group. The second group consists of natural mouse strains such as arrd2/arrd2 (Mdm gene mutation) and the senescence accelerated mice (SAM) which spontaneously develop features of dry AMD (68). The third group of mice was immunologically or mechanically manipulated by immunization with CEP (an oxidative fragment of DHA found in drusen), injection of vectors containing VEGF or peptides, or laser photocoagulation (3-4, 68). We have selected several representative mouse models for dry, wet and both forms for AMD to discuss the phenotype and disease mechanisms of AMD.

5.1. Dry AMD models

ABCR: ABCR, a retina-specific ATP-binding cassette transporter sub-family A member 4 (ABCA4), is a multiple-span transmembrane protein which is exclusively localized in the rim region of OS discs (69-70). It acts as a flipase for removing all-trans-retinal and its derivatives from the OS disc lumen to the cytoplasm for the reduction of all-trans-retinol for the visual cycle by using energy via ATP hydrolysis (71-73). Mutations in the ABCR gene cause a wide spectrum of retinal degenerative diseases including Stargardt macular degeneration (74) and AMD (75). The Abcr knockout mouse (Abcr-/-) was created by replacing 4.0 kb of the promoter region plus exon 1 by a PGK-neo cassette (71). Abcr-/- mice show delayed rod dark adaptation and recovery following light exposure with abnormal clearance of all-trans-retinol, which results in the accumulation of N-retinylidene phosphatidylethanolamine (N-ret-PE) and all-trans-retinal in the OSs (71-72). High levels of lipofuscin in the RPE cells which exhibit electron density bodies under electron microscopy and elevated levels of lipofuscin fluorophores, A2PE-H2 and A2E, are also seen in the RPE cells (71-72). Furthermore, higher levels of expression of oxidative stress genes and elevated lipid peroxidation, as well as elevated levels of complement-activation products and down regulation of complement-regulatory proteins (CRPs) in the Abcr-/- eyes were observed (41). These data suggested that A2E accumulation causes oxidative stress, complement activation and reduced CRPs levels, all of which induced chronic inflammation of the RPE (41). The progression of photoreceptor degeneration is very slow in the Abcr-/- mice (76). In albino background, Abcr-/- began photoreceptor cell loss at 8 months of age and worsened after that (77). Therefore the AMD phenotypic is due to the atrophy of RPE rather than photoreceptor degeneration in this mouse model.

ELOVL4: ELOVL4, an ER resident membrane-bound protein in the retina, belongs to the ELO (elongation of long chain fatty acid) family and participates in the biosynthesis of very long-chain fatty acids and docosahexaenoic acid (DHA) (78-79). Recent studies demonstrated that ELOVL4 is required for the synthesis of C28 and C30 very long chain saturated fatty acids (VLC-
FA) and of C28-C38 very long chain polyunsaturated fatty acids (VLC-PUFA) (80). Mutation of this gene results in loss of its retention in the ER with subsequent mislocalization as aggregates suggesting that the mutant protein is misfolded/unfolded and results in the UPR activation (81). The mutant protein is also unable to target to the ER for fatty acid biosynthesis (80) thus causing Stargardt-like macular dystrophy (STGD3) and atrophic macular degeneration (dry AMD). The transgenic mouse was created by introducing a 5-bp deletion of nucleotides (AACTT) at 790 –794 bp of the human wild type ELOVL4 gene (82). In the Elov4^-/- mice, high A2E levels were detected in the RPE cells at 2 months of age even in lines that express very low amounts of transgenic protein, and lipofuscin accumulates in the RPE at 7 months of age (83). Failure to form normal OS in the photoreceptors was seen in lines expressing higher levels of the transgene. The loss of 50% of photoreceptors occurs at 6 weeks, 16 weeks and 18 months in higher, middle and lower ELOVL4-expressing lines, respectively, correlated with decline in retinal function (82).

5.2. Wet AMD models

CNV: CNV models are commonly used for neovascularization development and therapeutic treatment for wet AMD. These animal models are induced by laser photocoagulation, surgical and genetically engineered (84). Laser-induced CNV disrupts BM and results in a high frequency (100%) of the development of CNV within 2–3 weeks (85). This model has the advantage that it is inexpensive, reproducible, relatively easy to create and the rapid onset of the CNV ensures the experiment can be processed and finished in a relatively short time period. However, the variable rate of CNV and transient CNV leakage are the major disadvantages of this animal model (84).

Vldlr: Transgenic Vldlr^-/- mouse, created by a partial deletion of exon 5 in the very low density lipoprotein receptor (Vldlr) gene on chromosome 19, is characterized by progressive and developmental neovascularization (86) and is a model for RAP (retinal angiomatous proliferation) in humans (87). In contrast to typical wet AMD in which the new blood vessels arise from the choroid extending to the subretinal space, the pathologic new blood vessels in RAP originate from the inner retina (outer plexiform layer) at postnatal day (P) 13–14, extend through the photoreceptors, invade the subretinal space and choroid, and eventually cause RPE disruption, BM exposure and photoreceptor degeneration with significant fibrosis (86-88). The development of abnormal leaky new vessels reaches the maximum at P42 (86-87). Decreased mRNA levels of rhodopsin and cone-opsin were detected around 2 months of age but retinal degeneration and ERG amplitude reduction occur late, at 3 months (89) and 6 months of age (90), respectively. Decreased levels of 11-cis-retinal and retinyl esters occurred at an old age (8 months) (89). Vldlr^-/- mice have severe retinal vascular leakage and impaired blood-retinal barrier (89), and exhibit up regulation of pro-inflammatory factors, adherent leukocytes and molecules as well as increased NF-kB expression (89, 91). In addition, significant higher levels of VEGF and bFGF, and GFAP (glial fibrillary acidic protein) were shown in the Vldlr^-/- mice (91-92). These observations suggest that up-regulation of inflammatory response factors and activation of MAPK/Akt/NF-kB signaling cascade, activation of Müller cells, along with elevated VEGF and bFGF expression, all contribute to neovascularization in the Vldlr^-/- mice (91). Furthermore, elevated LRP5/6 (low-density lipoprotein receptor-related protein 5 or 6) and free beta-catenin expression levels indicated that wt (wg/int1, wingless/integration 1) signaling pathway was activated in Vldlr^-/- mice and knockdown of the expression of Vldlr by siRNA proves that Vldlr is a negative regulator of the wnt signal pathway and a positive regulator of angiogenesis (92). Dickkopf-1 (wt pathway inhibitor which binds LRP5/6) treatment increases phosphorylated beta-catenin, and decreases VEGF and free beta-catenin levels demonstrating that Vldlr-deficiency causes VEGF overexpression and CNV through the wnt signaling pathway (92).

5.3. Both wet and dry AMD models

SOD1: Sod 1 (superoxide dismutase 1) deficient (Sod1^-/-) mice exhibit many features seen in wet AMD patients (93-94) such as progressive photoreceptor degeneration with drusen, thickened BM, and choroidal neovascularization in the older mice (94-95). ONL (outer nuclear layer) thickness at 10 weeks is not different from the WT (wild type); significant loss of ONL starts at 30 week and 50 weeks in INL (inner nuclear layer); the ultrastructure of the retina at 15 months old mice showed oxidative stress associated appearance including swollen nuclei, vacuolized cytoplasm, disrupted membranes and damaged mitochondria. ERG assessment of the retinal function before 40 weeks showed no difference from the WT, and both the rod and cone function showed no reduction at 40 weeks of age (95). The mice with the G86R mutation of SOD 1 gene exhibited a diminution of electroretinographic activity following exposure to constant bright light for 20 days and specific degeneration of photoreceptor cells (96).

Ccl2/Cx3cr1: The Ccl2^-/-/Cx3cr1^-/- double knockout transgenic mouse, a model characterized with lower fertility, develops early onset, at 6 weeks of age, of retinal lesions similar to human drusen. Other prominent features include abnormal RPE cells, thickened BM with drusen which are smaller than drusen in AMD patients, photoreceptor degeneration and significantly high levels of A2E accumulation, choroidal neovascularization and decreased levels of ER resident chaperone protein, ERP29, in the retina (46). When Ccl2^-/-/Cx3cr1^-/- mice were fed with either a high or low omega-3 LCPUFA diet, retinal lesions were observed at 6 weeks of age in both groups but the number of lesions was decreased in the mice that ingested a high omega-3 LCPUFA diet, compared to control group which developed RPE changes, progressive neovascularizations and drusen-like lesions as revealed by fundoscopy, histology and ultrastructure (46).

6. THERAPIES OF AMD

Because the pathogenesis of AMD is complicated and largely unknown, there is no effective and promising single treatment for prevention and/or cure. Destruction, or slowing the formation, of CNV by laser photocoagulation or anti-VEGF therapy have been shown to be the most successful treatment, but no proven therapy is available for RPE detachment, geographic atrophy, and early stage
AMD (2). Currently, ranibizumab (lucentis) and pegaptanib are two anti-VEGF agents approved by the US Food and Drug Administration (FDA) for treatment of neovascular AMD and they significantly improve the visual acuity of wet AMD patients. Bevacizumab (avastin), a commonly used anti-VEGF agent similar to ranibizumab, was shown to be effective for two years and was well tolerated and safe in most patients [see review (97-99)]. However, the treatment requires repeated dosing about once a month because neovascularization occurs through multiple signaling pathways (100) and frequent intravitreal injection could cause eye damage (2, 101). Multiple therapeutic strategies which address effectiveness, safety, cost, dosing and efficacy are being development including gene therapy, anti-angiogenic factors, and blockage of signaling pathways (102).

6.1. Pharmacological treatment
6.1.1. Antioxidant treatment

N-acetyl-cysteine, a precursor of glutathione and a potent antioxidant, was intraperitoneally injected into laser photoagulation-induced CNV mice and 4-HNE-modified protein and activation of NF-kB in nuclear extract were markedly suppressed after 3 hours and 6 hours respectively. Also, macrophage and neutrophil recruitment were inhibited, the levels of MCP-1, chemokine (C-X-C motif) ligand (CXL)-1, VEGF, and VEGF receptor (VEGFR) -1 were reduced, and the extent of CNV was significantly reduced by 7 days after treatment (103).

Lutein (LUT) and zeaxanthin (ZEA) are plant carotenoids that are present in a normal diet and major components in macular pigment (104-105). Increased dietary intake of LUT and ZEA resulted in increased plasma levels which were positively and significantly associated with macular pigment optical density (104). Pretreatment of rat retinal neurons in culture with DHA, LUT, ZEA or BC (beta-carotene) alone, or LUT, ZEA with DHA reduced oxidative stress-induced apoptosis in photoreceptors, preserved the mitochondrial membrane potential, cytochrome C translocation from mitochondria and enhanced photoreceptor development, survival and differentiation (105).

Sod1−/− mice treated with a mixture of antioxidants experienced a significant reduction in RNV, and wild type mice treated with the same drug also reduced RNV and CNV (93).

Zinc supplementation has prevented blindness in 25% of the patients with dry AMD by effectively decreasing oxidative stress and inflammatory cytokines (106).

Omega-3 fatty acids and their acid metabolites, resolvins and protectins, are endogenous anti-inflammatory compounds which can prevent NF-kB signaling, thus providing a putative target for AMD treatment (107). Ccl2−/−/Cx3cr1−/− mice were fed with either a high or low dose of long-chain omega-3 polyunsaturated (n-3) fatty acids (omega-3 LCPUFA). Although retinal lesions were observed at 6 weeks of age in both groups, the number of lesions was decreased in the mice that ingested a high omega-3 LCPUFA diet compared with the low n-3 fatty acids group (46, 108). And in the control group deprived of the omega-3 LCPUFA, the retinal lesion continued to progress (46).

α-tocopherol is a vitamin E component and is a major lipid-soluble chain-breaking antioxidant in mammals. Acrolein, a product of lipid peroxidation caused significant loss of ARPE-19 cell viability and a series of oxidative–associated reactions. Pretreatment of ARPE-19 cell with α-tocopherol activated the Keap1/Nrf2 pathway which increased the expression and/or activation of several phase II enzymes, consequently improving mitochondrial function, lowered ROS and protein oxidation levels and protected RPE cells from acrolein-induced damage while enhancing cell viability (109).

Melatonin has receptors localized in RPE cells and has the potential to prevent telomere shortening. Clinical trials showed that orally taking 3 mg melatonin each night for 3 months reduced pathologic macular changes in AMD patients (110).

Treating Abcr−/− mice with isoretinoin resulted in complete blockage of new synthesis of A2PE-H2, A2E and A2E-oxiranes and reduced lipofuscin accumulation (111). Vitamin A and fenretinide (an inhibitor of vitamin A) can significantly increase the levels of retinyl esters and reduce lipofuscin accumulation in the Abcr−/− mice (112).

6.1.2. Antiangiogenic treatment

Carboxyamidotriazole (CAI), an anti-angiogenic and anti-tumor agent, was formulated with aqueous beta-hydroxypropyl cyclodextrin (bHPCD-CAI) and intravitreally administered to a laser-induced CNV mouse model. Its effects on reduction of CNV lesion volume occurred in a dose-dependent manner. Pharmacokinetics demonstrated that there is a high concentration of CAI in the vitreous compartment without ocular toxicology (113). Antiangiogenic drugs such as lodamin CAI, CAI, or nerve growth factor receptor inhibitor K252a significantly regressed or inhibited laser induced CNV in mice (113-114).

Immune response depends on the constitutive expression of FasL, a death-inducing ligand, the expression of which inhibits inflammation and tumor growth by inducing apoptosis in cells (115). FasL expression in the RPE cells plays an important role in controlling neovascular diseases (115). Laser-induced CNV mice were injected intravitreally with recombinant soluble human FasL, or treated with doxycycline (an MMP inhibitor) in the drinking water. Both antiangiogenic agents resulted in prevention of neovascularization on day 7 (FasL) or day 1 and 3 (doxycycline) (116).

6.1.3. Various factor inhibitors

Insulin-like growth factor-1 is involved in ocular neovascularization. Its receptor (IGF-1R) inhibitor PPI (cyclotigian picipodophyllin) reduced the CNV area and...
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significantly decreased the VEGF levels in the choroid in laser-induced CNV mice (117).

Weekly intravitreal injections of compstatin (50 micro gram), a complement component 3 (C3) inhibitor, into 16-year old and 4-year old monkeys with dry AMD resulted in a diffusion of drusen in the macular region at 6 months after injection whereas monthly injections of 1 mg of compstatin resulted in the partial disappearance of drusen was observed by 9 months after injection (118).

Notch signaling occurs via a mechanism which plays a myriad of roles during vascular development and recent studies suggest that its regulation of angiogenesis is in concert with the VEGF pathway (119-121). VEGF receptor (VEGFR-2) is an important receptor tyrosine kinase which plays a premier role in the normal vascular development and neovascularization (122). Inhibition of Notch signaling (121) or VEGFR-2 expression by peptide vaccination (antigen peptide of human VEGFR-2) (123) reduced the CNV lesion volume to 18% and retinal vasculature leakage to 80%. Also, orally administered VEGFR-2 inhibitors significantly decreased the fluorescein leakage and the volume of CNV membrane (124-125).

6.2. Gene therapy

Overexpression of glutathione peroxidase 4 (Gpx4) in vivo by transgenic mice with inducible expression of Gpx4 in photoreceptors strongly protected retinal structure and function in oxidative damage-induced retinal degeneration. It also reduced oxidative stress induced RPE cell damage. Furthermore, overexpression of Gpx4 provided a better therapeutic benefit than overexpression of SOD1 or SOD2 although they are important endogenous defense systems (126).

Subretinal delivery of C3 expressing recombinant adenovirus (adenvC3) to adult murine RPE cells induced significant morphological changes which resemble features of AMD, such as significantly increased vascular permeability, endothelial cell proliferation and migration, and RPE atrophy (127).

RPE deterioration and CNV development require the activation of complement alternative pathway (CAP). Abnormalities of CAP regulation lead to the inflammation, drusen formation and the development of AMD. Complement factor H (CFH) is a CAP control protein. A recombinant form of CFH, CR2-CFH, containing the N-terminal domain of mouse CFH encoding CAP-inhibitor and linked to a complement receptor 2 (CR2) encoding a target fragment for C3 component binding, was demonstrated to reduce the loss of RPE integrity and decrease angiogenesis in CNV mice. Also, the expression of VEGF and C3 was significantly reduced and retinal function was restored to about 80% of that in the wild type mice (28).

Selective knockdown of ocular CFH in a laser-induced mouse CNV model resulted in increased MAC deposition and lead to the early onset and exacerbation of CNV (19). CD59 is a complement regulatory protein controlling the formation and function of MAC when it presents on the cell surface. It can bind to C8, C9, or both in the MAC complex and prevents the polymerization of C9 and the formation of MAC (128). CD59, with a membrane targeting moiety (APT542), can firmly bind to the cell surface. Treatment of laser-induced CNV mice with recombinant, soluble membrane-targeted CD59 (rCD59-AP542) (129) blocked the complement activation and the formation of MAC, reduced the size of fully developed CNV by 79%, increased apoptosis and decreased cell proliferation in the neovascular complex. Overexpression of human CD59 (hCD59) by delivering an adenovirus (Ad) vector into murine RPE cells (130) prevented MAC deposition and MAC-induced damage to RPE cells.

CCR3 (eosinophil/mast cell chemokine receptor) and its ligands eotaxin-1, -2, and -3 are specifically expressed in choroidal neovascular endothelial cells in humans with AMD. Blockade of CCR3 (anti-CCR3 neutralizing antibodies) or its ligands resulted in the suppression of CNV in a laser-induced CNV mouse model. Data demonstrated that this is due to direct inhibition of choroidal endothelial cell proliferation and is uncoupled from inflammation and independent of macrophage and neutrophil recruitment. Furthermore, it proved to be more effective than anti-VEGF-A treatment and is not toxic to the retina (131). However, immunostaining showed no specific expression of CCR3 in or near CNV indicating that CCR3 does not play a direct role in CNV development and questions the therapeutic approach of targeting CCR3 to suppress CNV (132).

AAV-mediated expression of a soluble form of VEGF receptor (Flt) 1 (AAV.sFlt 1) which only contained the extracellular domains of the Flt-1 protein, was subretinally delivered to trVEGF029 mice (133) or laser-induced CNV mice and monkeys (134). This lead to sustained sFlt expression for 8 months (mice) and 17 months (monkeys) and was accompanied by 8 months regression of CNV (134). The retinal neovascularization in the ONL was reversible; photoreceptor numbers and retinal function were retained (133-134). Similarly, AAV5.sFLT01, in which the Flt-1 signal peptide sequence was directly fused to Flt-1 domain 2 and linked to a human IgGi-Fc region was subretinally injected into Ccl2−/−/C3cr1−/− mice. This resulted in arrested retinal lesions, lowed A2E levels, reduced lipofuscin accumulation and decreased levels of ERK signals 3 months after treatment (135).

6.3. Regenerative medicine

During the past several years, stem cell-based therapeutic applications in retinal degenerative diseases have attracted more attention because of their potential for advanced treatment of diseases (136-137). Stem cells are a group of pluripotent cells which can enter the circulatory system and, when needed, differentiate into specialized cells (137) to generate various organs. Currently, many published reports indicate that stem cells from a variety of cell types can differentiate into photoreceptor cells or RPE cells as indicated by expression of key markers of these
ocular cells [see review (137)]. Compared to gene therapy, which is effective for the treatment of early stage diseases, the stem cells have the potential to treat advanced ocular diseases (136). The Ali research group reported in 2006, the transplantation of neural retinal cell suspensions from GFP (green florescent protein) transgenic (Nrl-GFP$$^{+/-}$$) mice at postnatal day 1, into the subretinal space of GFP-negative wild type littermates and adult recipients. The mixture of proliferating progenitors, post-mitotic precursors and differentiated cells that do not yet express the markers of mature photoreceptors, migrated into the retina and displayed the morphological features of mature photoreceptors three weeks later (138). Furthermore, they revealed that P3−5 donor cells from retina showed the greatest integration, but donor cells from other ages can only survive without integration. Three mouse models of inherited retinal degeneration: adult retinal degeneration slow (rds), P1 retinal degeneration fast (rd) and a 4 week rhodopsin knockout (rhor$$^{-/-}$$) were tested by subretinal injection. P1 Nrl-GFP$$^{+/-}$$ donor cells integrated into the retina of adult rds mice and remained viable for at least 10 weeks. Peripherin-2, which was absent in the adult retina of adult rds mice and remained viable for at least 10 weeks. Transplanted Nrl-GFP$$^{+/-}$$ cells integrated into the recipient ONL of rho$$^{+/-}$$ mice, and rhodopsin was immunolocalized to the outer segments (138). Moreover, the transplant cells were more sensitive to the light-evoked responses at relatively lower light intensities than the rho$$^{-/-}$$ shams injected eyes (138).

Stem cells have been isolated from adult tissues and shown to have the capacity of self-renewal and regeneration for ocular structures, thus providing many potential treatments for wet AMD (139-140). Human embryonic stem cell-derived RPE (hES-RPE) differentiate into functionally polarized hES-RPE cells as early as 4 weeks and had a normal chromosomal karyotype. Phenotypically polarized hES-RPE cells showed expression of RPE-specific genes, prominent expression of PEDF which was secreted into the medium and enhanced retinal progenitor cells (RPCs) survival (141). Adult bone-marrow-derived endothelial progenitor cells (EPCs) were reported to be capable of incorporating into the existing blood vessels after injection into the vitreous of new born mice and forming vascular mosaics and endogenous retinal vascular endothelial cells (142). Patients with AMD and mice with laser-induced CNV expressed high levels of collagenase-3 (MMP13), a matrix metalloproteinase (MMP) family member. A deficiency of MMP13 in mice was shown to inhibit the formation of laser-induced CNV suggesting that MMP13 is required for CNV. Several injections of mesenchymal stem cells (MSCs) from wild type bone marrow fully restored CNV indicating that these stem cells also participated in CNV (143). Engineered bone marrow-derived MSCs were intravenously injected into a laser-induced CNV mouse model and were specifically recruited into CNV lesions where they differentiated into multiple cell types and participated in the neovascular development. Furthermore they produced PEDF to inhibit the growth of CNVs and regression of CNV which were mediated by RPE cells (144). Bone marrow cells from enhanced GFP (EGFP) transgenic mice were transplanted into the wild type adult, three months later CNV was induced by photocoagulation, the vascular wall cells of the CNV expressed EGFP and CD31. This result demonstrated that bone-marrow-derived stem cells participated in the development of the neo-blood vessels in the CNV (145).

6.4. Nanomedicine

Nanoparticles, because of their tiny size and large surface area, have proven to be ideal carriers for effectively and efficiently delivering drugs, nucleotides and peptides to the eye (146-147). Some metal nanoparticles have shown themselves to be direct antioxidants and are able to block increases of ROS (148). Rare earth cerium oxide nanoparticles (nanoceria) have a high capacity to reverse oxygenation/deoxygenation without alteration of the fluorite lattice structure (149). They can exist as, and switch between, the $$+3$$ (fully reduced) and $$+4$$ (fully oxidized) valence states in a redox reaction. This results in their ability to scavenge free-radicals due to an increase in oxygen vacancies in the surface of the crystal structure and by the loss of oxygen and/or its electrons (149-150). The unique characteristics and capabilities of nanoceria, such as biocompatibility, small size, and cell/nuclear membrane permeability, have proven them to be excellent agents for biological applications (151-153). Nanoceria exhibit catalytic activities of the two antioxidative enzymes superoxide dismutase and catalase (154), and they act as direct antioxidants to inhibit ROS-induced death of a variety of cells in vitro and in vivo (151, 154-159). Nanoceria have no genotoxicity, are not toxic to cultured cells and are non pathologic to mouse tissues (154, 156-159). Due to their unique regenerative properties, nanoceria do not require repeat dosages as seen with the use of dietary supplements of other antioxidants.

Our lab has shown that nanoceria can be stored in a phosphate buffered saline at room temperature for a couple of years and still maintain their stability and effectiveness (unpublished data). Almost 80% of nanoceria injected intravitreally are retained in the albino rat retina for at least 4 months as determined by inductively coupled plasma-mass spectrometry (ICP-MS) (159). In addition, nanoceria have been shown to prevent photoreceptor death and to rescue retinal function in light-damaged rats (151, 155). They also slow photoreceptor degeneration and improve retinal function in an inherited retinal degenerative mouse model (tubby). This mouse has a splicing mutation in the Tub gene and is a phenotypic model of Usher syndrome in humans. The nanoceria protection occurs by up regulating survival signaling pathways and/or down regulating apoptosis signaling pathways (160). In addition, nanoceria were successfully employed to inhibit developing neovascularization in the Vldlr$$^{-/-}$$ mice when intravitreally administered before the disease onset (P7) (161). Their effect on down regulation of VEGF levels is concentration-dependent with a total dose of 172 nanograms in 1 micro liter providing the highest benefit (unpublished data). By preventing increases in ROS, nanoceria inhibit VEGF
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Figure 1. Nanoceria prevent abnormal development of pathologic blood vessels in the retina (top row) and choroid (bottom row) at P28 after single injection at P7. All retinal blood vessels were labeled green by the vascular filling assay. Wild type (WT) retina shows web-like vasculature (A) and choroid shows no choroidal “tufts” (D) whereas Vldlr-/- retina exhibits numerous intraretinal vascular “blebs” (arrows) (B) and Vldlr-/- choroid has many bright “tufts” (E). A single intravitreal injection of nanoceria (CeO2) at P7 greatly inhibits the appearance of these “blebs” (C) and “tufts” (F). G and H show the quantitative analyses of IRN blebs and SRN tufts from this set of the experiment. Data were from nine animals, three at each of the three developmental ages (P14, P21and P28) with or without nanoceria. *p<0.05; **p<0.01. This figure (from Zhou et al 2011) was reproduced with the permission from PLoS One.

expression, prevent the development and maintenance of abnormal “leaky” intraretinal blood vessels (RNV) and inhibit choroidal neovascularizations (CNV) (Figures 1-3). They also decrease the levels of oxidative stress indicators (ROS, NADPH oxidase, nitrotyrosine, 8-hydroxydeoxyguanosine) (Figure 4). These effects were still present 3 weeks after injection (161) without adverse toxicity or other side effects. Nanoceria also can down regulate VEGF expression levels and regress the existing vascular lesions in the Vldlr-/- mice when injected at P28 and analyzed at P35 (Figure 5) (161). These data demonstrated that nanoceria are effective in the treatment of pathologic neovascularizations in a mouse model of AMD and most likely will be as effective in humans with AMD, diabetic retinopathy (DR), retinopathy of prematurity (ROP) and other neovascular ocular diseases.

7. CONCLUSION AND PERSPECTIVES

AMD is a very complex and multifactor ocular disease. It has two clinical forms and has a wide variation in pathological development. In late stages of disease development, the dry form can change to the wet form and this phenomenon can also occur during treatment. Extensive efforts have been made to discover the etiology of AMD using molecular, cellular, biochemistry, physiology and animal models, yet the mechanisms underlying AMD are not fully known and will require further experimentation. We think, that because excessive production of ROS is a common node for degenerative diseases of the retina and because the rise in ROS occurs upstream of almost all other ocular pathology in AMD, it represents the “Achilles Heel” of AMD. Because nanoceria are catalytic antioxidants which are retained in the retina for months and act as a mixture of broad spectrum antioxidants to limit ROS and ROS-mediated damage, we think nanoceria can at least slow the progression of AMD and preserve vision in current patients with AMD. The combinatorial use of nanoceria with other therapeutic agents may lead to the most effective treatment
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Figure 2. Nanoceria reduce the VEGF expression during the development of the Vldlr^-/- retina. Western blots show that VEGF protein levels are significantly higher in the Vldlr^-/- (v/v) retina than the wild type (WT) at P14 and P28 (A & B) and the increase is age-dependent. The developmental increases of VEGF levels are not altered by saline injection but nanoceria (CeO2) injection at P7 significantly reduced the VEGF levels with maximum decreases of 5 fold (C & D). *p<0.05; **p<0.01. This figure (from Zhou et al 2011) was reproduced with the permission from PLoS One.

Figure 3. Nanoceria inhibit the ectopic expression of VEGF in the ONL of the Vldlr^-/- retina at P28. Photomicrographs of immunolocalization of VEGF in the Vldlr^-/- retina showed that WT retinas (A, B) had very low levels of VEGF in the ONL whereas discontinuous heavy staining of VEGF was located in the ONL of Vldlr^-/- (C, D). The labeling was greatly reduced in the nanoceria (CeO2) injected (E, F) Vldlr^-/- mice. DAPI (blue) was used to visualize the nuclei. A, C, E, 20x; B, D, F, 40x. This figure (from Zhou et al 2011) was reproduced with the permission from PLoS One.
Figure 4. Nanoceria reduce oxidative stress in the Vldlr<sup>-/-</sup> retina. Eyes at P28 from wild type (left panel), Vldlr<sup>-/-</sup> injected with saline (middle panel) or nanoceria (CeO<sub>2</sub>) (right panel) were sectioned and proceed for immunocytochemistry to visualize the distribution of oxidative stress markers for ROS (DCF), NADPH-oxidase (P47-phox), Nitrotyrosine and DNA damage (8-OHdG). Wild type shows either no labeling (A) or very low levels (D, G, J) of these markers, Vldlr<sup>-/-</sup> retina exhibits very high labeling (B, E, H, K) while nanoceria treatment greatly reduced the labeling of these markers (C, F, I, L). This figure (from Zhou <i>et al</i> 2011) was reproduced with the permission from PLoS One.

Figure 5. Retinal vascular lesions in the Vldlr<sup>-/-</sup> retinas require continual production of excess ROS. Vldlr<sup>-/-</sup> mice were injected at P28 with saline or nanoceria and killed one week later on P35. Analysis of VEGF levels by western blots (A) showed a four-fold reduction (B) within one week of nanoceria injection. The numbers of IRN blebs (C), and SRN tufts (D) were also dramatically reduced. *p<0.05; **p<0.01. This figure (from Zhou <i>et al</i> 2011) was reproduced with the permission from PLoS One.
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for wet and dry AMD and other eye diseases which progress through the production of ROS.

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