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RPE Necroptosis in Response to Oxidative Stress and in AMD

Jakub Hanus, PhD[1], **Chastain Anderson**[1], and **Shusheng Wang, PhD**[1,2,*]

[1]Department of Cell and Molecular Biology, Tulane University, New Orleans, LA, 70118, USA

[2]Department of Ophthalmology, Tulane University, New Orleans, LA, 70118, USA

Abstract

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in the elderly. The underlying mechanism of non-neovascular AMD (dry AMD), also named geographic atrophy (GA) remains unclear and the mechanism of retinal pigment epithelial (RPE) cell death in AMD is controversial. We review the history and recent progress in understanding the mechanism of RPE cell death induced by oxidative stress, in AMD mouse models, and in AMD patients. Due to the limitation of toolsets to distinguish between apoptosis and necroptosis (or necrosis), most previous research concludes that apoptosis is a major mechanism for RPE cell death in response to oxidative stress and in AMD. Recent studies suggest necroptosis as a major mechanism of RPE cell death in response to oxidative stress. Moreover, ultrastructural and histopathological studies support necrosis as major mechanism of RPE cells death in AMD. In this review, we discuss the mechanism of RPE cell death in response to oxidative stress, in AMD mouse models, and in human AMD patients. Based on the literature, we hypothesize that necroptosis is a major mechanism for RPE cell death in response to oxidative stress and in AMD.

Keywords

Apoptosis; Age-related macular degeneration; Geographic atrophy; Necroptosis; Necrosis; Oxidative stress; RPE

1. Oxidative stress and inflammation in AMD pathogenesis

Age-related macular degeneration (AMD) is a degenerative disorder of the macula, the region of the central retina responsible for the greatest visual acuity. AMD is the leading cause of irreversible blindness in the elderly in the western world. In the USA alone, about 1.8 million individuals are afflicted with the disease, and that number is projected to reach ~3 million by 2020 (Friedman et al., 2004). About 20% of legal blindness can be attributed to AMD, and it's projected that nearly 80 million people worldwide will be affected by AMD by the year 2020 (Biarnes et al., 2011). Early stage AMD is associated with the

*Corresponding author: S Wang, Department of Cell and Molecular Biology and Ophthalmology, Tulane University, 2000 Percival Stern Hall, 6400 Freret Street, New Orleans, LA 70118, USA. Tel: 504 862 3163; Fax: 504 865 6785; swang1@tulane.edu.

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pathological deposits (drusen) between the Bruch's membrane and the retinal pigment epithelium (RPE). Late AMD has non-neovascular (known as 'dry') and neovascular (known as 'wet') forms. Neovascular AMD accounts for 10% of the cases but results in sudden and acute vision loss in patients due to choroidal neovascularization. There are several treatment options available for neovascular AMD using anti-angiogenic agents (Brown et al., 2006; Rosenfeld et al., 2006; Zampros et al., 2012). Late stage non-neovascular AMD, also called geographic atrophy (GA), is characterized by scattered or confluent areas of degradation of RPE cells, as well as the overlying photoreceptors and the underlying choriocapillaris. It accounts for 90% of AMD cases and is currently untreatable.

AMD is a multi-factorial disease with unclear etiology. Age is the most consistent risk factor associated with AMD, but genetic factors, oxidative stress and inflammation are also significant contributors to AMD pathogenesis (Cai and McGinnis, 2012). Cigarette smoking, which induces systemic oxidative stress, has been demonstrated to be a significant risk factor for AMD. Consistently, clinical studies have shown that the progression of AMD can be slowed with antioxidant vitamins and zinc supplements (Age-Related Eye Disease Study 2 Research, 2013; Age-Related Eye Disease Study Research, 2001). For reviews of oxidative stress and AMD, refer to Jarret and Boulton (Jarrett and Boulton, 2012) and Mettu et al (Mettu et al., 2012).

The retina represents one of the highest oxygen-consuming tissues in the human body (Yu and Cringle, 2005). Intensive oxygen metabolism, continual exposure to light, high concentrations of polyunsaturated fatty acids, and the presence of photosensitizers increase the production of reactive oxygen species (ROS) in the retina (Beatty et al., 2000; Khandhadia and Lotery, 2010). ROS overproduction by chronic oxidative stress can exceed the anti-oxidation capability of the retina and lead to modification and damage of carbohydrates, membrane lipids, proteins, and nucleic acids. An age-related increase in lipofuscin (potent photoinducible ROS generator in RPE), 8-Oxoguanine (a major product of oxidative DNA damage), mtDNA damage, carboxyethylpyrole (CEP, an oxidation fragment of docosahexaenoic acid), and the presence of 4-Hydroxynonenal (4-HNE) and Malondialdehyde (MDA, products of lipid peroxidation) have been observed in the ageing retina (Jarrett and Boulton, 2012). In the retina, photoreceptor (PR) cells are consistently exposed to light and oxygen, and are thus particularly susceptible to oxidative damage. Among other important functions, RPE cells are required for PR outer segment membrane phagocytosis, and therefore critical for PR survival, function and renewal. Consequently, RPE degeneration caused by oxidative stress or other stresses usually causes secondary PR cell death. In AMD, oxidative stress works in concert with other risk factors such as: aging, smoking, phototoxicity, and genetic factors, leading to sub-RPE drusen deposits, RPE/PR cell death, and the resultant inflammatory and immune responses. These processes may aggravate oxidative stress and inflammation, forming a vicious cycle leading to AMD pathogenesis. In support of a critical role for oxidative stress in AMD, modified oxidative products, such as CEP and MDA, have been shown to cause inflammatory response and retinal phenotype in animal models similar to that in AMD (Hollyfield et al., 2008; Suzuki et al., 2007).

The complement system is a component of the human immune system that helps to protect against pathogens. It consists of over 40 proteins and cell surface receptors and is generally divided into three pathways: the classical or innate, the lectin, and the alternative pathway that primarily involves Factor H (Donoso et al., 2010). Factor H is primarily synthesized in the liver and is a key regulator of the alternative pathway. It is recognized by cell surface membranes and protects host cells from the rapid and progressive destruction of other activated components of the complement system. Factor H may also be synthesized locally, particularly in those tissues that have localized self-turnover such as the eye, brain, kidney, liver, and vascular organs (Mandal and Ayyagari, 2006). Complement factor H (CFH) polymorphism (Y402H) is strongly associated with AMD (Edwards et al., 2005; Haines et al., 2005; Klein et al., 2005; Scholl et al., 2005).

Interestingly, a recent study showed that CFH protects from the oxidative stress-induced inflammation by binding to MDA (Weismann et al., 2011), supporting interactions among genetic factor, oxidative stress and inflammation in AMD. Besides oxidative stress, other factors may also lead to RPE cytotoxicity. In a recent report, pathogenic RNA species (*Alu* and DsRNA) were shown to trigger RPE cytotoxicity and cause GA in mice (Kaneko et al., 2011; Murakami et al., 2013). In GA patients, *Alu* RNA is abnormally accumulated due to the down-regulation of its processing enzyme Dicer, and has been shown to induce mitochondrial ROS in RPE cells (Kaneko et al., 2011; Tarallo et al., 2012).

2. Overview of cell death pathways

Oxidative stress is known to induce RPE cell death both *in vitro* and *in vivo*. However, the nature of RPE cell death and its implication in AMD, especially in GA, has recently fallen into controversy, although it is generally accepted that PR cells die from apoptosis in AMD. Because of the critical role of the RPE in AMD, deciphering the mechanism of RPE cell death in AMD will not only be crucial to understanding AMD (especially GA) pathogenesis, but also instrumental for designing targeted therapeutic agents for AMD. In this section, we will provide an overview of the pathways leading to cell death, with a focus on apoptosis and necrosis. The features that can distinguish these two cellular processes will be discussed.

2.1. Apoptosis: Extrinsic and intrinsic pathways

Apoptosis was first described in 1972 (Kerr et al., 1972) and is a tightly regulated process of programmed death that is crucial for normal embryonic development and tissue homeostasis. Impaired apoptosis can lead to tumor development, autoimmune, or neurodegenerative diseases (Nagata et al., 2003). Apoptosis inhibits immunological response and does not affect neighboring cells due to cell debris being phagocytosed by macrophages or other surrounding cells (Jacobson et al., 1997; Zhang and Xu, 2000). Morphologically, apoptosis is characterized by cytoplasm shrinkage, chromatin condensation, nuclear fragmentation, formation of apoptotic bodies, and maintenance of the plasma membrane. It involves caspase activation, mitochondrial outer membrane permeabilization, DNA fragmentation, and lysosomal membrane permeabilization. Depending on cell type and insult, apoptosis can be executed by different pathways.

The extrinsic pathway involves cellular membrane receptors; and intrinsic pathway is mediated through mitochondria (Rossi and Gaidano, 2003) (Fig. 1). Both pathways share activation of caspase cascade. Caspases are cysteine proteases that are activated by autocatalysis or proteolysis by other caspases. They are divided into two groups: initiator caspases (Caspase-8, -9, -10) and effector caspases (Caspase-3, -6 and -7), the latter of which lack the Death Effector Domain (DED). The effector caspases lead to the cleavage or degradation of cellular substrates including inhibitory subunit of endonuclease DNA fragmentation factor-45 (DFF45/ICAD), poly (ADP-ribose) polymerase (PARP), histones, nuclear lamins, keratin, actin, and kinases. DFF protein, together with other nucleases, is involved in genomic DNA degradation. The catalytic subunit of DFF endonuclease (DFF40/CAD) is both a deoxyribonucleotide-specific and a double-strand-specific nuclease, and introduces DNA double strand breaks into chromatin substrates generating characteristic 180–200bp apoptotic ladder (Hanus et al., 2008). The extrinsic apoptotic pathway is initiated by membrane receptors (death receptors) and their ligands. Death receptors belong to the Tumor Necrosis Factor (TNF) family. The best characterized ligands and receptors include FasL/FasR, TNFa/TNFR1 and TNFR2. Activation of these receptors triggers their trimerization. These activated trimers then recruit TNF-receptor 1-associated death domain protein (TRADD) protein followed by Fas associated death domain (FADD) protein, inducing the formation of a death inducing signaling complex (DISC). In the DISC complex, FADD protein recruits initiator Caspase-8 and -10 via homotypic death domain interactions, which in turn activates the downstream effector caspases (caspase-3, -6 and -7), leading to substrate cleavage and apoptotic cell death. In addition, caspase-8-mediated cleavage of the BH3-only protein Bid amplifies the death receptor-induced cell death program by activating the mitochondrial (intrinsic) pathway of apoptosis (Harper et al., 2003; Rossi and Gaidano, 2003; Wajant et al., 2003).

The intrinsic apoptotic pathway can be activated by growth factor deprivation, DNA damage, gamma radiation, UV radiation, excessive ROS levels, virus infection, or oncogene activation. It is a major pathway leading to caspase cascade activation through release of cytochrome c from mitochondria. This pathway is initiated by activation of the proapoptotic Bcl-2 family proteins (Bid, Bim, Bad and Bmf) followed by activation Bax subfamily of Bcl-2 proteins (Bax, Bak and Bok) localized on outer mitochondrial membrane. Bax activation causes opening of mitochondria transition pores that leads to release of cytochrome c to the cytoplasm. In the presence of ATP, cytochrome c binds to apoptotic protease activating factor (Apaf) - 1 protein and forms complex known as apoptosome. Apoptosome in turn recruits and activates Caspase-9 and causes subsequent activation of effector caspases (Du et al., 2000; Eskes et al., 2000; Green, 2000).

2.2. Necrosis

Necrosis used to be considered a passive type of cell death induced by physical, chemical, and/or biological insult. The fundamental features of necrosis include: cellular energy depletion, damage to membrane lipids, and loss of function of homeostatic ion pumps/channels. Major morphological changes that occur in necrosis include: cell swelling; formation of cytoplasmic vacuoles; distended endoplasmic reticulum; formation of cytoplasmic blebs; condensed, swollen, or ruptured mitochondria; disaggregation and

detachment of ribosomes; disrupted organelle membranes; swollen and ruptured lysosomes; and eventually disruption of the cell membrane. The nucleus disintegrates late and in some cases chromatin condensation occurs.

Recent studies have found that necrosis is mediated by receptor interacting protein kinases (RIPK), leading to its renaming as necroptosis (Christofferson and Yuan, 2010) (Fig. 2). Necrosis shares the upstream TNFR pathway with apoptosis, specifically at the level of DISC formation. RIPK3 has been shown to play a central role in necrosis, while RIPK1 is critically involved in both necrosis and apoptosis (Declercq et al., 2009; Zhang et al., 2009). Necrosis is normally inhibited since RIPK1 and RIPK3 are cleaved by caspase 8. The delicate balance between RIPK1/RIPK3 stabilization and caspase 8-driven RIPK1/RIPK3 cleavage seems to function as the switch between apoptosis and necrosis. RIPK3 acts upstream to phosphorylate RIPK1, which in turn mediates downstream RIPK3 phosphorylation. Both RIPK3 and kinase activity of RIPK1 are essential for the formation of the stable RIPK1-RIPK3 complex, called the necrosome (Cho et al., 2009). Upon induction of necrosis, RIP3 kinase was shown to be recruited to the lipid rafts of the plasma membrane, and form a transient complex with TRAF2 protein (Shen et al., 2004). RIPK1 and RIPK3 are also known to form an amyloid structure through their RIP homotypic interaction motifs. This hetero-oligomeric amyloid structure is a functional signaling complex that mediates programmed necrosis (Li et al., 2012).

Mixed Lineage Kinase Domain-Like (MLKL) and mitochondrial phosphatase 5 (PGAM5) were recently identified as key RIPK3 downstream components of TNF-induced necrosis (Sun et al., 2012; Wang et al., 2012). Phosphorylation of MLKL on its kinase domain by RIPK3 is required for MLKL to function in necrosis (Sun et al., 2012). The recruitment of MLKL by RIPK3 leads to phosphorylation of MLKL and its tetramerization. Oligomerization of MLKL leads to the translocation of MLKL complex to lipid rafts of the plasma membrane. It has been proposed that function of MLKL relies on its oligomerization and plasma membrane association (Hildebrand et al., 2014) and targeting the plasma membrane by MLKL is a critical step in the execution of necrotic cell death (Chen et al., 2014). Two forms of PGAM5: PGAM5L and PGAM5S bind sequentially to RIPK1-RIPK3-MLKL complex. The binding of the necrosome to PGAM5S marks its translocation to more hydrophobic environment mitochondrion, and leads to dephosphorylation and activation of Drp1 protein (Wang et al., 2012). Drp1-mediated mitochondrial fragmentation seems to be a common required step for apoptosis and necrosis.

During necrosis, mitochondria degenerate and subsequently cluster around nuclei before nuclear and cytoplasmic membrane breakdown. Besides mitochondrial fragmentation, mitochondrial permeability transition pore (mPTP) opening is also critically involved in necrosis. Initially identified as a key component for apoptosis, Bcl-2 family members Bax/Bak were recently shown to also be involved in necrosis. During apoptosis, Bax and Bak generate large pores allowing the permeability of the mitochondrial outer membrane, leading to cytochrome c release and the subsequent apoptotic caspase activation. In Ca^{2+} overload-induced necrosis model a key regulated event is the opening of mPTP, a protein complex that was proposed to span the inner and outer mitochondrial membranes in facilitating the swelling and rupture of the mitochondria. Cyclophilin D (CypD), a

peptidylprolyl isomerase located in the mitochondrial matrix, is critical for necrosis through binding and regulating the opening of the mPTP in inner membrane (Baines et al., 2005; Nakagawa et al., 2005). CypD dissociation from ADP-ATP translocase (ANT), a component of mPTP, allows for the opening of mPTP and the subsequent ATP depletion and ROS increase potentially through a RIPK3-dependent mechanism (Tian et al., 2013). Bax/Bak are required for mPTP-dependent necrosis by serving as a necessary functional component of the mPTP within the outer mitochondrial membrane in a manner that is distinct from their more active mechanism of oligomerization during apoptosis, placing Bax/Bak at the bifurcation point of mitochondrial-dependent apoptosis and necrosis (Karch et al., 2013).

2.3. Comparison between apoptosis and necrosis

Although apoptosis and necrosis have distinct features, they also share common features, such as: chromatin condensation, mitochondrial permeability, and DNA degradation. Here we summarize the features of apoptosis and necrosis (Table 1 and Figure 3), and highlight the point that any separate method may not be sufficient to unequivocally distinguish between apoptosis and necrosis. Distinguishing necrosis from apoptosis should not be based on either morphological or biochemical criteria alone, but rather should take into account and integrate all available data (Krysko et al., 2008). When apoptosis is inhibited, necrosis may become an alternative route for cell death. Caspase-8 is a switch between apoptosis and necrosis (O'Donnell et al., 2011). In apoptosis, RIPK3 is cleaved by caspase-8, therefore preventing the induction of necrosis. RIPK3 is normally uniformly distributed throughout the living cell, and aggregates to form a discrete punctate pattern after induction of necrosis (Sun et al., 2012).

For example, a traditional TUNEL assay cannot distinguish between necrosis and apoptosis because both types of cell death feature chromatin degradation. Apoptotic DNA degradation is highly organized and visible on an agarose gel as DNA ladder (180bp DNA and its multiples). Necrotic DNA degradation is not organized and partial visible as a smear on agarose gel. Both types of cell death are characterized by nuclear shrinkage, but in apoptosis chromatin condensation and fragmentation is well defined.

Apoptotic cells are propidium iodate (PI) negative until the very late stage, while necrotic cells are PI-positive at very early stage. However, necrotic cells may show Annexin V-positive/PI-negative staining before they become PI-positive. Furthermore, both processes show mitochondria membrane permeabilization and are phosphotidylserine (PS) positive shown by Annexin V staining. Apoptosis shows outer mitochondrial membrane (OMM) permeabilization while necrosis shows the permeabilization of both OMM and IMM (inner mitochondrial membrane). Apoptosis requires several ATP-dependent steps, and intracellular ATP levels remain largely unchanged until the very end of the process, while necrosis occurs under intracellular ATP depletion. High Mobility Group (HMG) proteins are nuclear proteins and are essential part of chromatin structure. During necrosis, HMGB1 protein is passively released from the nucleus and secreted to the extracellular matrix where it promotes inflammation (Scaffidi et al., 2002). During apoptosis, HMGB1 is tightly bound to DNA and sequestered inside apoptotic bodies along with chromatin and cellular organelles (Ellerman et al., 2007). Moreover, it is commonly believed that apoptosis is anti-

inflammatory and tolerogenic while necrosis triggers inflammation and immune response. Necrotic cells can release multiple pro-inflammatory factors, including heat shock proteins and HMG proteins (such as HMGB1), to activate inflammatory response (Zitvogel et al., 2010).

3. Necrosis or apoptosis: nature of oxidative stress-induced RPE cell death

Because of the involvement of oxidative stress and RPE cell death in AMD (especially GA) numerous research has been focused on studying the mechanism of oxidative stress-induced RPE cell death as an approach to decipher the mechanism of AMD pathogenesis. An established, *in vitro* system to investigate oxidative stress-induced RPE cell death and AMD is to treat RPE cells with hydrogen peroxide (H_2O_2) or tert-Butyl hydroperoxide (tBHP). H_2O_2 is a non-radical ROS produced in living cells as a result of cell metabolism. It can directly damage DNA, lipids, and other macromolecules causing oxidative injury to the cell. When not metabolized, H_2O_2 can convert to extremely reactive hydroxyl radical (•OH) via the Fenton reaction leading to the propagation of the oxidative damage to the cell. Similarly, t-BHP can decompose to other alkoxyl and peroxyl radicals in a reaction aided by metal ions that can generate ROS, including H_2O_2. Unlike H_2O_2, tBHP evokes consistent cellular stress.

Using either an H_2O_2 or tBHP model, researches attribute oxidative stress-induced RPE cell death mostly to apoptosis. A wide range of H_2O_2 (50μM to 2.5mM) concentrations and treatment durations have been used in these studies. Barak et al used TUNEL assays as well as PI/Annexin V staining to detect RPE apoptosis/necrosis in response to H_2O_2 (0.5–2.5 mM) exposure for 16 to 24 hours (Barak et al., 2001). PI-negative/annexin V-positive cells were counted as early apoptotic cells; PI-positive/annexin V–positive cells were considered to be late stage apoptotic cells; and PI-positive/annexin V–negative cells were counted as necrotic cells. They concluded that both H2O2 at 1 mM or tBHP at 0.3 mM induced mostly apoptosis and H_2O_2 at 2.5mM induces mostly necrosis. Alge et al analyzed caspase-3 activation by measuring the cleavage of its substrate DEVD-p-nitroaniline (DEVD-pNA) and found a 3.5 fold increase of Caspase-3 activity by 300μM H_2O_2, while overexpression of B-crystallin reduced caspase-3 activity and RPE cell death (Alge et al., 2002). Strunnikova et al showed that, in response to prolonged oxidant agent hydroquinone (HQ), ARPE-19 cells showed non-apoptotic (50Kb) DNA laddering, arguing against classical apoptosis under this condition (Strunnikova et al., 2004). Similar nuclear DNA degradation to 50kb fragments was also observed during RPE cell death when exposed to menadione (Zhang et al., 2003). Kaarniranta et al found that 4-hydroxynonenal (HNE)-derived oxidative stress reduced cellular viability, which is associated with caspase-3 independent apoptosis (Kaarniranta et al., 2005). Morphologically, slight rounding and swelling of a few cells were seen after the exposure to 30 μM HNE, suggesting necrosis in those cells. However, a later study by Sharma et al showed that 4-HNE induces p53-mediated apoptosis in RPE cells and activates caspase-3 (Sharma et al., 2008). Therefore more work is needed to confirm the nature of RPE cell death under this condition. The superoxide dismutase (SOD) family functions as a major component of antioxidant systems by converting superoxide to H_2O_2. Kasahara et al isolated primary cultures of RPE cells from wild-type, heterozygous *Sod2* knockout mice, and hemizygous *Sod2* mice with overexpression of the

Sod2 enzyme (Kasahara et al., 2005). Oxidative stress was induced in these cells by exposing them to H_2O_2 (0–500 μM) for 1 hour and re-culturing them in normal medium for various durations (0–24 hours). Apoptosis in the RPE was detected by TUNEL staining, mitochondrial transmembrane potential (MTP) measurement, and cytochrome c leakage from mitochondria. They concluded that SOD2 protects against oxidation-induced apoptosis in mouse RPE cells. Ho et al found that exposure to a lethal dose of H_2O_2 (1mM) in RPE cells elicited Bax translocation to the mitochondria and release of apoptosis-inducing factor (AIF) from the mitochondria, both of which were prevented by either JNK- or p38-specific inhibitors (Ho et al., 2006). Sreekumar et al reported that in response to 150μM H_2O_2 treatment, activated caspase-3 was not detected unless methionine sulfoxide reductase (Msr) A was silenced in RPE cells (Sreekumar et al., 2005). Similarly, Glotin et al reported that caspase-3 was not activated in RPE cells in response to tBHP (Glotin et al., 2006). However, caspase-9 was activated as early as after 10 min tBHP treatment and over 2 hour treatment periods. Moreover, transient ERK1/2 activation was observed, and inhibition of MEK1/2 completely suppressed ERK1/2 activation and blocked RPE apoptosis induced by tBHP. Cai et al examined RPE death in response to tBHP (300μM) using a combination of assays, including TUNEL assay, Annexin V staining Western blot for pro-caspase-3, cytchrome c, and PARP, as well as measuring mitochondrial membrane potential (Cai et al., 1999). They found that RPE cells showed positive TUNEL and Annexin V staining and cytoplasmic cytochrome c expression, but decreased level of pro-caspase-3, PARP and lost mitochondrial membrane potential after tBHP treatment. Interestingly apoptotic DNA degradation was not observed.

There are also a few studies suggested necrosis as a major mechanism for oxidative stress-induced RPE death. Kim at al proposed that H_2O_2 induced-RPE cell death is a combination of apoptosis and necrosis (Kim et al., 2003). H_2O_2 at 400uM was shown to induce early apoptosis accompanied by condensed and fragmented nuclei. Increase of H_2O_2 concentration resulted in bigger onset of late apoptotic and necrotic cell death, while concentration above 700μM induced mostly necrotic cell death. By transmission electron microscopy, chromatin condensation and marginalization were shown in RPE cells treated with 500μM H_2O_2, and organelle swelling and membrane rupture were seen in cells treated with 600μM H_2O_2. However, in a recent report by Li et al, H_2O_2 at 400μM was able to cause massive cell death associated with typical features of necrosis: a swollen cell body, ruptured membrane, and condensed nuclei (Li et al., 2010). Overload of intracellular calcium concentration was observed and considered to be an early step of necrotic RPE cell death. Our laboratory systematically studies the nature of RPE cell death in response to oxidative stress. We observed typical necrotic hallmarks like PI membrane permeability, RIPK3 activation or HMGB1 release from the nucleus. Consistently, caspase inhibitor z-VAD did not affect H_2O_2 (or tBHP)-induced RPE death. However, RIPK1 inhibitors necrostatins significantly rescued RPE from oxidative stress-induced cell death. Furthermore RIPK3 knockdown with siRNA increased survival of RPE cells after H_2O_2 or tBHP treatment (Hanus et al., 2013).

Taken together, conflicting results exist regarding the mechanism of RPE cell death induced by oxidative stress, mainly due to the reliance on single or very limited methods to identify

apoptosis versus necrosis. Nevertheless, most morphological studies support a necrotic nature of RPE death. However, biochemical methods used for identifying apoptosis, including TUNEL assay, and Annexin V/PI staining, are not sufficient to distinguish apoptosis from necrosis. The conflicting data on caspase-3 activation in RPE cells in response to oxidative stress mostly result from the failure of the antibodies in detecting activated caspase-3. As to the mechanism for the lack of apoptosis in RPE cells, one hypothesis is that RPE cells are postmitotic cells and therefore have limited apoptotic potential (Kaldarar-Pedotti, 1979). Postmitotic cells, such as neurons, cardiomyocytes, and skeletal myotubes, have been shown to have limited regeneration potential and restricted apoptotic potential (Wright and Deshmukh, 2006). Caspase-8 has been shown to be a molecular switch between apoptosis and necrosis induced by death receptors (Lin et al., 1999). Caspase-8 suppresses RIP3–RIP1 kinase complex-dependent necroptosis by cleavage of RIPK3 when cells undergo TNF-induced apoptosis (Christofferson et al., 2013). Indeed, Yang at al recently showed that RPE cells have low levels of caspase-8 expression (Yang et al., 2007). ARPE-19 cells are unable to undergo TNFα-induced apoptosis due to low levels of caspase-8 at both the mRNA and protein level. Restoring caspase-8 expression using adenovirus made ARPE-19 cells susceptible to TNFα-induced apoptosis. DFF is a heterodimeric complex composed of a 40-kDa subunit (DFF40/CAD) that mediates regulated chromatin condensation and DNA fragmentation in response to apoptotic signals, and a 45-kDa subunit that is a specific molecular chaperone and an inhibitor of DFF40 (Widlak and Garrard, 2005). DFF40/CAD is released and activated upon the cleavage of DFF45/ICAD by caspase-3. Compared to the high level DFF expression in HeLa cells, DFF protein expression was barely detectable in RPE cells regardless of H_2O_2 treatment (Hanus et al., 2013). Low caspase-8 and DFF protein expression in RPE cells indicates an impairment of the apoptotic pathway in RPE cells, consistent with our hypothesis that RPE cells die mainly from necrosis.

4. Inflammatory nature of oxidative stress-induced RPE cell death

RPE cell damage and death caused by oxidative stress, other stresses, or alternative complement pathway activation, have been proposed to trigger the release of intracellular and extracellular damage-associated molecular pattern (DAMP) molecules. DAMP can induce immune and inflammatory response and lead to AMD progression (Qin and Rodrigues, 2008). Apoptosis is anti-inflammatory and tolerogenic while necrosis triggers inflammation and immune response. Our hypothesis that RPE cells die from necrosis in response to oxidative stress is consistent with the notion that RPE cells promote inflammatory processes in the retina during AMD development. HMGB1 is a major DAMP molecule passively released from nucleus during necrosis and secreted to extracellular matrix and promotes inflammation (Scaffidi et al., 2002). We found that HMGB1, which induces the expression inflammatory gene TNFα in RPE cells and in macrophages, is secreted from necrotic RPE cells (Hanus et al., 2013). Moreover, when HMGB1 antibody was used to deplete HMGB1 in the medium, the induction of TNF-α expression by the conditioned medium was blunted, suggesting a critical role for HMGB1 in inducing inflammatory gene expression by necrotic RPE cells. Consistently, in a recent publication using a mouse model of dsRNA-induced retinal degeneration, RIPK3-dependent release of

HMGB1 to the vitreous is correlated with RPE necrosis and TNFα and IL-6 production (Murakami et al., 2013). These results indicate RPE cell necrosis in response to oxidative stress has a detrimental role in inflammation.

As mentioned earlier complement activation is associated with AMD. It was found that C-reactive protein (CRP) levels appear to be higher in AMD patients (Seddon et al., 2004). When the CRP function is blocked, complement attack induces necrotic RPE cell death (Yang et al., 2011). CRP also recruits complement factor H (CFH) to necrotic lesions and blocks release of proinflammatory cytokines. CFH risk variant (Y402H) has reduced ability to bind CRP which results in complement activation and induction of inflammation (Lauer et al., 2011).

Additionally, the H384 variant of complement factor H (CFH) binds DNA and necrotic cells better than Y384 which potentially could influence complement activation and oponization in some areas of drusen (Sjoberg et al., 2007). Those evidence suggest a function for complement pathway in clearing necrotic RPE cells, which establishes a correlation between RPE necrosis, complement activation, and local inflammation, which can explain at least part of the AMD pathogenesis.

5. RPE cell death in animal models of non-neovascular AMD and GA

A variety of animal models have been developed to investigate the etiology of AMD, especially non-neovascular AMD and GA (Ramkumar et al., 2010). The models include: models of complement factor pathway (*Cfh*$^{-/-}$ and CFH(Y402H) transgenic mice), inflammatory gene models (*Ccl2*$^{-/-}$, *Ccr2*$^{-/-}$, *Cx3cr1*$^{-/-}$ and *Ccl2*$^{-/-}$/*Cx3cr1*$^{-/-}$ mice), sodium iodate injection model, oxidative gene/damage models (*Sod1*$^{-/-}$ mice, *Sod2* knockdown mice, *Nrf2*$^{-/-}$ mice, CEP-MSA immunized mice), lipid/glucose metabolism models (*ApoE*$^{-/-}$ mice; apoE2 and apoE4 knockin mice; *mcd/mcd* and *mcd2/mcd2* transgenic mice), and pathogenic RNA models (*Alu* RNA or dsRNA injection). These models display some but not all features of AMD. Since the mouse doesn't have a macula, the data from any mouse model may not be directly translatable to human AMD. Nevertheless, these models should be informative regarding the mechanisms of human AMD. Here we will focus on the ultrastructure of the RPE and neighboring tissues in oxidative stress mouse models and other relevant models, with the hope of illuminating the nature of RPE death in these models.

The SOD family is a major component of the antioxidant system. SOD1 is found principally in the cytoplasm, SOD2 is located in mitochondrial matrix, and SOD3 is extracellular. *Sod1*$^{-/-}$ mice have features of typical AMD in humans. Senescent *Sod1*$^{-/-}$ mice displayed drusen, thickened Bruch's membrane, and choroidal neovascularization (Hashizume et al., 2008). Vacuolization, one of the common morphological hallmarks of necrotic cells, was observed in the RPE cells of *Sod1*$^{-/-}$ animals. Interestingly, TUNEL-positive cells were not significantly different between *Sod1*$^{-/-}$ and wild-type mice, and activated caspase-3 could not be detected in the retina of *Sod1*$^{-/-}$ mice, arguing against apoptosis in this model. Similarly, RPE hypopigmentation, vacuolization and atrophy were observed in a ribozyme AAV virus generated *SOD2* knockdown mouse model (Justilien et al., 2007). NRF2 is a

master regulator of many antioxidant/detoxification genes (Zenkov et al., 2013). *Nrf2*$^{-/-}$ mice develop age-dependent RPE degeneration, spontaneous CNV, and subretinal inflammatory protein deposits (Zhao et al., 2011). The RPE cells were highly vacuolated with membranous debris. Intermediate structures of autophagy, such as autophagosomes and autolysosomes, were readily detectable by electron microscopy. Carboxyethylpyrrole (CEP) is a unique oxidation fragment of docosahexaenoic acid found in AMD drusens and in plasma samples from AMD patients (Crabb et al., 2002; Gu et al., 2003). CEP-MSA immunized mice were recently established as a model for studying geographic RPE atrophy (Hollyfield et al., 2008). Features of RPE necrosis, including vesiculation, swelling, cell lysis, and nuclear pyknosis were observed. Additionally, the presence of monocytes in the inter-photoreceptor matrix was detected, supporting an inflammatory outcome of oxidative stress-induced necrosis. These data are consistent with our report that necrotic RPE cells induced by oxidative stress secrete the DAMP molecule HMGB1 and induce inflammatory gene expression.

Besides these models, injection of the oxidizing agent sodium iodate ($NaIO_3$) has also been used as an acute oxidative stress model for retinal degeneration. $NaIO_3$ is thought to possesses selective toxicity on RPE cells (Noell, 1953). It induces RPE disruption, atrophy, and significant retinal thinning (Wang et al., 2014). NaIO3 induces oxidative stress in RPE cells, and has been postulated to react with melanin, increasing its ability to convert glycine into glucoxylate, which damages cells by reacting with macromolecules (Baich and Ziegler, 1992; Behnam et al., 2006; Juel et al., 2013; Poldelski et al., 2001). $NaIO_3$ damages mostly the central pole of the retina but also affects the peripheral area, which resembles the clinical sequence of AMD pathogenesis (Machalinska et al., 2010). The effect of $NaIO_3$ in retina degeneration models is variable depending on the routes of administration and range of concentrations used. Intraperitoneal injection of $NaIO_3$ at 100mg/kg, intravenous injection of $NaIO_3$ at 25mg/kg, or retroobital injection of $NaIO_3$ at 40mg/kg have each shown to cause RPE damage that precedes photoreceptor apoptosis (Enzmann et al., 2006; Kiuchi et al., 2002; Zhou et al., 2014). RPE cell death was described as necrosis based on analysis of ultrastructure that revealed karyolysis, swollen cytoplasm, and the negative TUNEL staining of RPE cells. RPE damage was associated with macrophage infiltration and degeneration of photoreceptors through apoptosis (Zhou et al., 2014). Other research showed that even low concentration of $NaIO_3$ (15 mg/kg) were able to mimic the patchy loss of the RPE seen in RPE dystrophies and in GA (Franco et al., 2009). *In vitro* studies also led to similar conclusion, that high dose of $NaIO_3$ (500–1000 μg/ml) induced predominantly RPE cell necrosis (Zhou et al., 2014). Taken together, a majority of the studies support that RPE cells undergo necrosis *in vivo* in response to $NaIO_3$, which is followed by photoreceptor apoptosis and thinning of the retina. The molecular markers for RPE necrosis by $NaIO_3$ are still to be established.

Other AMD models that are potentially relevant to oxidative damage have also been reported. Iron is essential for cells, but ferrous iron (Fe2+) can cause oxidative damage via the Fenton reaction. Ceruloplasmin (Cp) and Hephaestin (Heph) are multicopper feroxidases that facilitate iron export. Mice with a combined deficiency of *Cp* and *Heph* (*Cp*$^{-/-}$/*Heph*$^{-/Y}$ mice) were found to have age-related iron accumulation, secondary increases in ferritin, and

retinal degeneration with AMD-like features (Hadziahmetovic et al., 2008; Hahn et al., 2004). RPE hypopigmentation, hypertrophy, hyperplasia, and necrosis were observed in 6–9 month double knockout mice, and subretinal macrophage infiltration was evident in 12 month-old mice. Cathepsin D (CatD) is an aspartic protease that is involved in the lysosomal digestion of the outer segments in the retina (Rakoczy et al., 1997). Homozygous transgenic mice (*mcd/mcd*) that express a form of CatD lacking the CatD cleavage sites showed RPE hypopigmentation, hyperpigmentation and hypertrophy, as well as drusen-like lesion (Rakoczy et al., 2002). An increase in apoptotic photoreceptors was reported in *mcd2/mcd2* mice with additional deletions in the catD cleavage sites (Zhang et al., 2005). These mice displayed earlier signs of retinal degeneration than the *mcd/mcd* mice. Three month-old *mcd2/mcd2* mice showed focal areas of RPE disorganization, clumping, proliferation, and RPE attenuation, depigmentation, and atrophy.

CFH polymorphisms have been strongly associated with AMD (Edwards et al., 2005; Haines et al., 2005; Klein et al., 2005; Scholl et al., 2005). Aged $Cfh^{-/-}$ mice showed thinning of Bruch's membrane, disorganization of rod photoreceptor outer segments, and changes in the distribution of RPE cell organelles (Coffey et al., 2007). CFH (Y402H) transgenic mice showed accumulation of subretinal cells that stained with macrophage/microglia marker, indicating local inflammation (Ufret-Vincenty et al., 2010). Basal laminar deposits, long-spaced collagen, and increased numbers of lipofuscin granules were also observed. Features of RPE apoptosis or necrosis were not documented these two models. However, CFH Y402 variant has shown better protective capacity in the clearance and removal of RPE necrotic debris and reduction of inflammation, establishing a link between CFH, oxidative stress, and RPE necrosis in AMD (Haines et al., 2005; Lauer et al., 2011).

Ccl2 and its receptor Ccr2 are essential for monocyte recruitment and mediate the adhesion of inflammatory cells to the vessels. Both $Ccl2^{-/-}$ and $Ccr2^{-/-}$ mouse models were found to have features of AMD (Ambati et al., 2003). $Ccl2^{-/-}$ and $Ccr2^{-/-}$ mice showed drusen-like subretinal deposit and thickening of Bruch's membrane after 9 months of age. RPE cells became swollen, and vacuolated with accumulation of high electron density bodies. At 16 months of age, RPE cells from $Ccl2^{-/-}$ mice had marked vacuolization with a degenerative nucleus and few pigment granules. RPE swelling and vacuolization suggest RPE necrosis in these models. Retinal microglial cells express the CX3C chemokine receptor 1 (CX3CR1). Homozygosity of the CX3CR1 M280 allele and V249I allele, associated with impaired microglial cell migration, has been shown to increase the risk of AMD (Combadiere et al., 2007; Tuo et al., 2004). $Cx3cr1^{-/-}$ mice displayed photoreceptor degeneration and subretinal deposit of microglial cells and lipid containing cells. The $Ccl2^{-/-}/Cx3cr1^{-/-}$ mouse model demonstrated early onset of the AMD phenotype with high penetrance (Tuo et al., 2007). $Ccl2^{-/-}/Cx3cr1^{-/-}$ mice began exhibiting drusen-like lesions by fundoscopy as early as 4–6 weeks of age. Bruch's membrane thickening, RPE hypopigmentation, depigmentation, vacuolization, and increased lipofuscin were observed. The RPE histology appears to be independent of the confounding *rd8* background, which is present in the reported $Ccl2^{-/-}/Cx3cr1^{-/-}$ mice (Chu et al., 2013; Mattapallil et al., 2012).

Apolipoprotein (APOE) polymorphisms provide a significant risk for AMD: the APOE ε4 allele confers a decreased AMD risk; the APOE ε2 allele is associated with a slightly

increased AMD risk (Baird et al., 2006; Klaver et al., 1998). *ApoE*$^{-/-}$ mice have elevated total plasma cholesterol and VLDL, and show vacuolated electrolucent vesicle in the Bruch's membrane (Dithmar et al., 2000). Aged *apoE3* knockin mice showed minor RPE vacuolization only on a high-fat diet (Malek et al., 2005). However, aged *apoE2* and *apoE4* knockin mice on a high-fat diet had a more severe phenotype with RPE vacuolization, hyperpigmentation and hypopigmentation, and Bruch's membrane thickening. These mouse models combine three AMD risk factors: advanced age, high fat cholesterol-rich diet, and APOE genotype, representing an excellent model for AMD. Although not specified, vacuolization of the RPE suggest necrotic cell death in these models.

Recent studies have indicated the involvement of pathogenic double strand RNAs (dsRNA), including *Alu* RNAs, in RPE cell death and geographic atrophy. *Alu* RNAs are dsRNAs about 300 nucleotides in length and are transcribed from Alu elements, the most common noncoding, repetitive DNA sequence in the human genome. Accumulation of *Alu* RNAs was observed in the RPE cells of GA eyes (Kaneko et al., 2011). *Alu* RNA overexpression reduced RPE cell viability and induced RPE degeneration in mice. Mechanistically, *Alu* RNA activates the NLRP3 inflammasome and triggers TLR-independent MyD88 signaling via IL18 in the RPE (Tarallo et al., 2012). Caspase-1, a component of the NLRP3 inflammasome, was shown to be activated by *Alu* RNA, and be critical for *Alu* RNA-induced RPE degeneration. Caspase-1 can trigger pyroptosis, a form of cell death characterized by formation of membrane pores and osmotic lysis (Fink and Cookson, 2006). However, glycine, a cytoprotective agent which attenuates pyroptosis, failed to inhibit *Alu* RNA induced RPE death, suggesting *Alu* RNA-induced RPE degeneration doesn't occur via pyropotosis. In an independent recent study, dsRNA analog poly(I:C) was shown to induce necrosis of the RPE cells as well as macrophage infiltration into the outer retina in the mouse. In *Ripk3*$^{-/-}$ mice, both necrosis and inflammation induced by poly(I:C) were prevented (Murakami et al., 2014). Moreover, decreased expression of pro-inflammatory cytokines (such as TNF-α and IL-6) in the retina, as well as attenuated intravitreal release of DAMP molecule HMGB1, was observed in *Ripk3*$^{-/-}$ mice. Although necrosis normally doesn't involve caspase-1 activation, based on these results it would be curious to test whether *Alu* RNA-induced RPE cell death occurs via necrosis. In this regard, *Alu* RNA has been shown to induce mitochondrial ROS in RPE cells. If this holds true *in vivo*, it would be consistent with our hypothesis that oxidative stress-induced RPE necrosis is a major mechanism of RPE death in AMD.

RPE cell swelling and vacuolization exist in multiple mouse models of AMD, especially in oxidative stress related models. This supports the hypothesis that necrosis is a major mechanism for RPE death in AMD. However, further work is required to unequivocally prove the necrotic nature of RPE death in these mouse models.

6. RPE cell death in human AMD patients

The mechanism of RPE death in human AMD patients has been controversial, with opinions split between apoptosis and necrosis (Dunaief et al., 2002; Farkas et al., 1971; Hageman et al., 2001; Xu et al., 1996). Most of the research was done before necrosis was characterized as a regulated pathway of cell death. These data were not derived from large sample sizes,

nor based on information gathered from multiple approaches (i.e., histology, ultrastructure, immunostaining, and molecular studies). Moreover, because of the slow progression of AMD (up to 5–15 years), it is difficult to track the spontaneous and isolated RPE death events during the course of AMD development. Therefore negative data regarding RPE death should be approached with caution. Nevertheless, here we will review the relevant references showing ultrastructural and molecular changes of RPE cell death in AMD patients.

Earlier clinical-pathological studies have defined the RPE alterations in GA (Rudolf et al., 2013; Sarks et al., 1988; Sarks, 1976). These features can be classified as follows (Rudolf et al., 2013): 0=uniform pigmentation and morphology; 1=Non-uniform but still epitheliod morphology and pigmentation change; 2A=Rounding and sloughing of individual cells from the underlying substrate, and anterior migration of cells within the subretinal space; 2B=Pigmented cellular fragments within basal laminar deposit; 2L=Double layer of continuous RPE; 3=anterior migration through the external limiting membrane and into neurosensory retina; 4=Loss of pigmented cells with persisting basal laminar deposits; 5=Loss of both pigmented cells and basal laminar deposit. Of note, grades 0 and 1 are considered normal aging, and in grades 4 and 5, many RPE cells are already not present. However, RPE rounding, sloughing, and entrapment of RPE-derived fragments within basal deposits are seen in grade 2 samples, supporting necrosis as a mechanism for RPE cell death in AMD, especially GA. Consistent with the inflammatory nature of necrosis, multi-nucleate giant cells have been shown to be present at the edge of the area of atrophy and the breakdown of Bruch's membrane, usually associated with clumps of pigment (Penfold et al., 1985, 1986). These giant cells are closely associated with macrophage-series cells, possibly representing the fusion of macrophages. These results support that AMD has a chronic inflammatory component, likely resulting from RPE necrosis.

In the past, TUNEL assay has been widely used to define apoptosis *in vivo*. Xu and colleagues have studied the role of apoptosis in human retinal degenerations (Xu et al., 1996). They examined 16 eyes diagnosed with AMD by histopathological criteria, and found RPE atrophy, drusen and irregularly thickened Bruch's membrane in 12 of the eyes. Interestingly, only two of the atrophic AMD samples showed photoreceptor apoptosis by TUNEL assay. Fragmentation and margination of the chromatin in photoreceptor was occasionally noted. However, RPE apoptosis was not noted in the atrophic AMD samples. Moreover, apoptosis of photoreceptors but not RPE cells was also observed in myopic macular degeneration and retinal detachment secondary to choroidal melanoma. On the contrary, Dundaief et al analyzing dying cells in GA retinas and found TUNEL-positive RPE and photoreceptor cells at edges of atrophic areas, suggesting apoptotic RPE cells in those AMD patients (Dunaief et al., 2002).

Taken together, although ultrastructural and histopathological studies support the hypothesis that necrosis is a major mechanism of RPE cells death in AMD, especially GA, further work is warranted to confirm the hypothesis by using a combination of recently developed toolsets for distinguishing apoptosis and necrosis. These include, examining membrane permeability, cellular ATP level, RIPK3 activation, and HMGB1 release.

7. Concluding Remarks

Based on the review of the nature of RPE cell death in response to oxidative stress *in vitro*, in non-neovascular AMD and GA mouse models, as well as the clinical-histological studies of human AMD patients, we propose a hypothesis that necrosis is a major mechanism of RPE cell death in AMD. The necrotic nature of RPE is consistent with following facts: A. RPE cells are postmitotic, and therefore have limited apoptotic potential; B. Necrosis can induce an inflammatory and immune response, but apoptosis normally doesn't; C. AMD is a disease in which chronic inflammation plays a critical role. Our hypothesis is supported by the following observations: A. Morphological evidence: necrotic features such as cell swelling, rounding, and vacuolization have been observed in *in vitro* models of oxidative-stress induced RPE death, AMD mouse models, and AMD (especially GA) patients. However, key features of apoptosis, such as active caspase-3 and apoptotic chromatin condensation, were not consistently reported; B. Molecular evidence: there is a shortage of molecular evidence that can distinguish between necrosis and apoptosis in *in vitro* or *in vivo*. TUNEL assay has been used to define apoptosis in the above-mentioned models. However, because of the existence of partial DNA degradation in necrosis, it is now accepted that TUNEL assay is not sufficient for distinguishing apoptosis from necrosis. We have provided compelling evidence that RPE cells die from necrosis in response to oxidative stress using an array of different approaches. Lack of apoptotic DNA fragmentation, caspase-3 cleavage, and failure to maintain intracellular ATP level in these cells argue against apoptosis as a major mechanism for RPE death in response to oxidative stress. RIPK3 activation and aggregation, HMGB1 release from the nucleus and secretion, and rescue of oxidative stress-induced RPE death by RIPK1 inhibitor necrostatins and by RIPK3 silencing strongly support necrosis as a major type of cell death in RPE cells in response to oxidative stress. Future work is warranted to rigorously test whether necrosis is a major type of cell death in animal models of AMD and AMD patients.

Although oxidative stress and RPE necrosis are the focus of this paper, other types of cell death and other types of stress also exist, and potentially influence AMD pathogenesis. For example, besides apoptosis and necrosis, autophagy and pyroptosis are also linked to cell death. Autophagy is a catabolic process aimed at degrading damaged organelles, proteins and cellular debris by engulfing them into a double membrane vesicle called the autophagosome and eliminating them by posterior fusion with the lysosome (Flores-Bellver et al., 2014). Overactive autophagy may be cytotoxic (Murrow and Debnath, 2013). Autophagy is characterized by the formation of the autophagosome containing lipidated LC3. Although not fully characterized, pyroptosis is a proinflammatory programmed cell death and is uniquely dependent on caspase (Fink and Cookson, 2005). Although our data suggest that autophagic cell death and pyroptosis are not major types of cell death in RPE cells in response to oxidative stress (Hanus et al., 2013), we should not rule out a role for these types of cell death in AMD. Although oxidative stress is a major type of stress involved in AMD pathogenesis, other stresses can also induce RPE death. For example, dsRNA was recently shown to induce RPE necrosis *in vivo* (Murakami et al., 2013), and *Alu* RNA was shown to induce caspase-1 dependent cell death, likely not pyroptosis (Tarallo et

al., 2012). The nature of RPE cell death by other stressors, such as lipofuscin, light or DNA damage, remains to be established.

Based on our hypothesis, we propose a model for AMD pathogenesis (Figure 4). In this model, oxidative stress and other stresses (i.e., pathogenic dsRNA species) can induce RPE dysfunction and necrosis, which in turn enhance drusen deposits, complement pathway activation, local inflammation, and immune response. These lead to more self-perpetuating RPE cell death through a neighbor-killing mechanism (Kaneko et al., 2011), or retraction-from-free-edge mechanism (Gallagher-Colombo et al., 2010), causing GA. The significance of our model lies not only in providing a mechanism for AMD pathogenesis, but also allaying a foundation for potential targeted therapy for AMD. Preventing RPE necrosis could prove to be beneficial for AMD patients at different stages. Two recent studies support this potential approach: RIPK1 inhibitor necrostatin-1 combined with caspase inhibitor z-VAD, was shown to inhibit photoreceptor necrosis induced by retinal detachment in mice (Trichonas et al., 2010); and dsRNA-induced RPE necrosis is prevented in *RIPK3* deficient mice (Murakami et al., 2013). For anti-necrosis approach to work, it would be important to establish the fate of RPE cells when necrosis is inhibited in AMD, and if they can still maintain their function in retinal homeostasis especially phagocytosis of photoreceptors. Reinforcement of RPE anti-oxidant repair mechanism or its combination with anti-necrosis therapy should also be considered in the future.

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Highlights

- Published data suggest programmed necrosis is a major mechanism of RPE death in AMD
- Inflammatory and immune response can be associated with RPE necrosis
- In *in vitro* model oxidative stress induced RPE cell death is RIPK1/RIPK3 dependent
- Use of multiple assays is needed to properly distinguish apoptosis from necroptosis

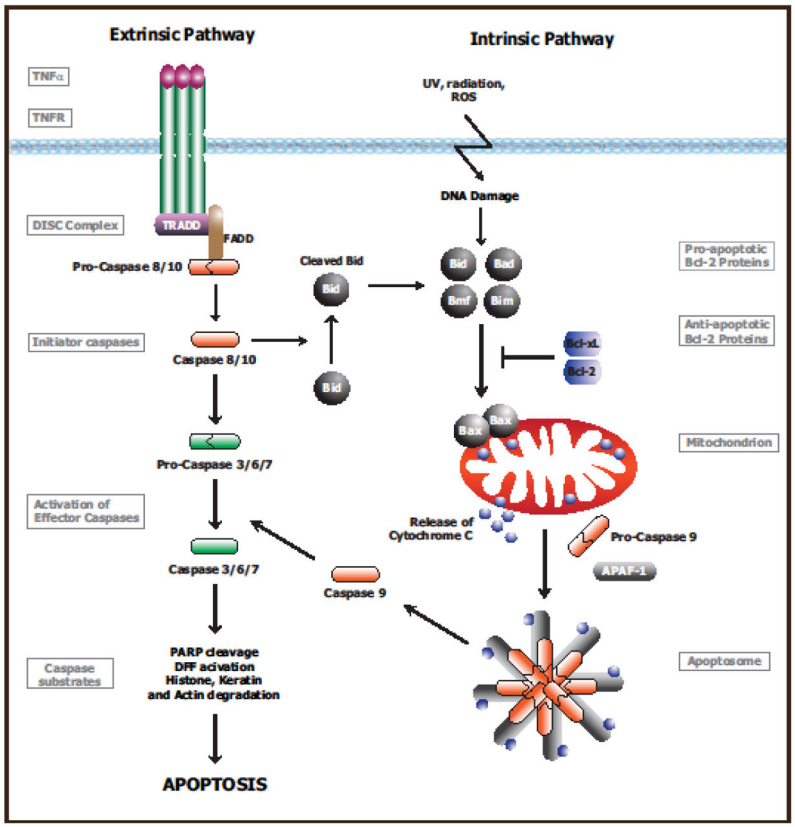

Figure 1. Overview of the apoptotic intrinsic and extrinsic pathways

Extrinsic apoptotic pathway is induced by activation of the membrane death receptors that leads to activation of the effector caspases and triggers caspase cascade that leads to cleavage of the caspases final targets, among the others: nuclear lamins, DFF45, PARP. Intrinsic pathway is triggered by DNA damage, gamma radiation, UV radiation, excessive ROS levels, virus infection or oncogenes activation. Activation of Bcl2 proteins leads to release of cytochrome c from mitochondrial intermembrane space leads to formation of apoptosome that induces activation of caspase-9 and effector caspase.

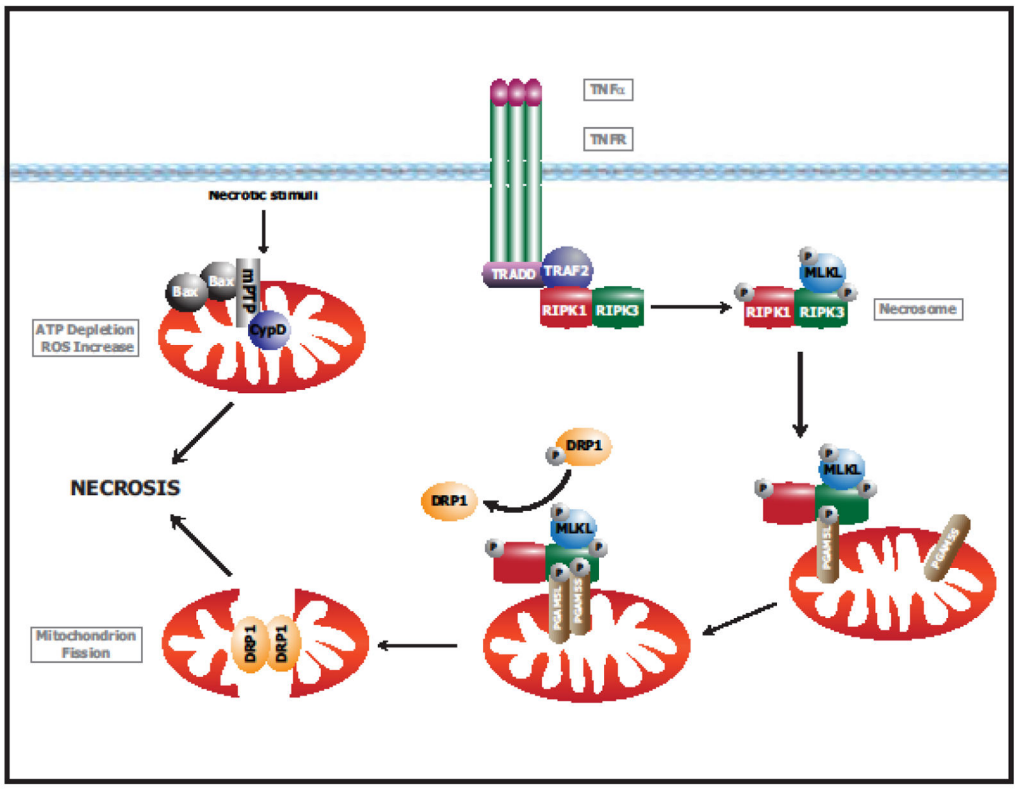

Figure 2. Overview of the necrotic pathways

Activation of TNF receptor leads to activation of RIP1 and RIP3 kinases when caspase-8 is not present or inactive. Autophosporylations of RIP1 and RIP3 leads to formation on necrosome, a complex that initiates necrotic signaling pathway. Necrosome recruits MLKL protein and assembles signaling complex at the membrane rafts. Recruitment of the PGAM5 marks translocation of the complex to hydrophilic environment and attachment to the mitochondrial membrane and activation of Drp1 protein that leads to mitochondrial fission and cell death. In the necrosis triggered by calcium overload, opening of mPTP leads to decrease of mitochondrial membrane potential, collapse of ATP production and release of ROS and triggering of necrosis in RIP3 dependent manner, although the signaling pathways leading to that event are unknown.

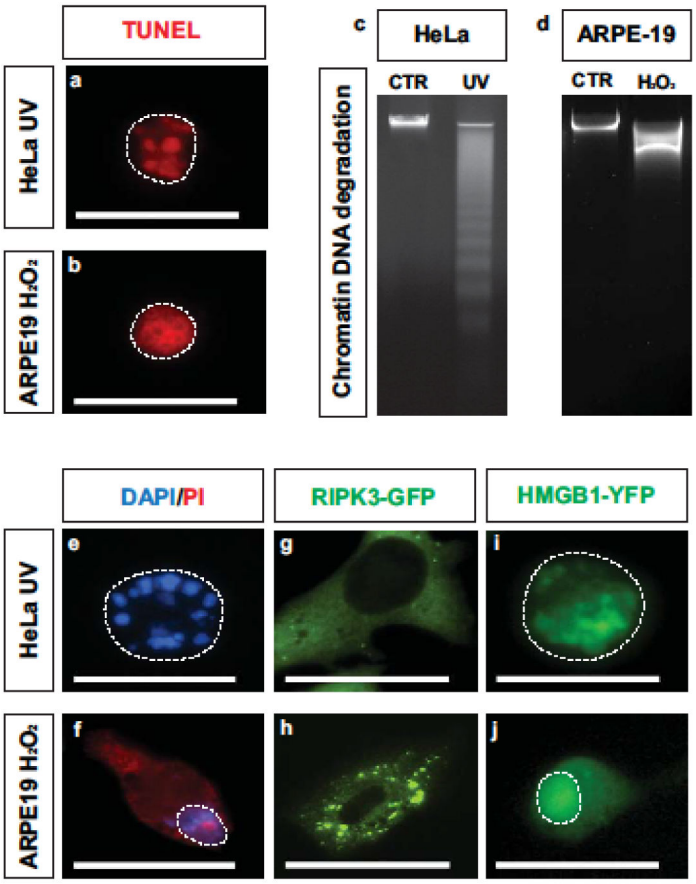

Figure 3. Comparison of apoptotic and necrotic hallmarks

To compare features of apoptosis and necrosis we exposed HeLa cells to UV irradiation (80J/cm^2) and ARPE-19 cells to hydrogen peroxide (300μM). Analysis of DNA degradation revealed that both apoptotic HeLa cells (a) and necrotic ARPE-19 cells (b) are TUNEL positive 24 hours after induction of cell death. Although both cell types have different nuclear morphology. Additionally analysis of chromatin degradation by electrophoresis reveals organized pattern of DNA degradation known as apoptotic ladder in HeLa cells (c), while in ARPE-19 unorganized DNA degradation is visible as a smear (d). DAPI staining shows strictly orchestrated process of nuclear fragmentation in apoptotic HeLa cells (e) which is revealed in nuclear morpholoty. Additionally cell membrane of the apoptotic cells remains intact throughout the whole process therefore it is resistant to propidium iodate. Necrotic cells lose membrane integrity early and it can be crossed by PI (f) and nucleus often shrinks and disintegrates. RIP3 kinase is a switch between apoptosis and necrosis. In apoptotic cells RIP3 kinase is degraded by caspase-3 (g, picture taken one hour after exposing HeLa cells to UV irradiation, points of RIP3 aggregation are not apoptosis specific and due to RIP3 overexpression), in necrosis RIP3 kinase forms distinct punctuations reflecting its activation and formation of the necrosome (h, picture taken 1 hour after exposing ARPE-19 cells to 300μM H_2O_2). HMGB1 is a chromatin structural protein, in apoptosis it binds tightly to DNA and is packed together with DNA into apoptotic bodies reflecting chromatin fragmentation pattern (i). During necrosis HMGB1 is passively

released from the nucleus to the cytoplasm, due to DNA modifications, through compromised nuclear envelope (j). RIP3 kinase was overexpressed as fusion protein RIPK3-GFP. HMGB1 protein was overexpressed as HMGB1-YFP fusion protein. Scale bar represents 25μm. Dashed line marks the region corresponding to the nucleus.

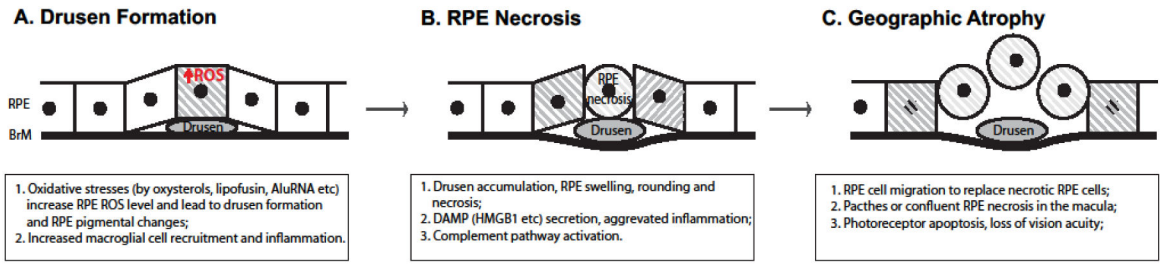

Figure 4. Model of AMD pathogenesis

RPE cells are exposed to highly oxidative environment shuffling waste from photoreceptors, and providing trophic support. As the eye ages cholesterol starts to accumulate on Bruch's membrane, and lipofucsin packets accumulate in RPE cells. Growing drusen attract macrophages and recruit choroidal dendritic cells while drusen accumulate products of lipid peroxidation they start to increase oxidative pressure on RPE cells (A). Damaged RPE cells die from necrosis, contributing to drusen growth, promoting inflammatory response by releasing DAMP molecules and promoting damage of the neighboring RPE cells (B). Those events attribute to self-perpetuating RPE cell death events (C).

Table 1

Comparison of biochemical features of apoptosis and necrosis.

	Apoptosis	**Necrosis**
Similarities		
Annexin V staining	Positive	Positive
PI staining	Positive in late apoptosis	Positive
Cytochrome c	Released from mitochondria	Released from mitochondria
DNA degradation	Organized DNA degradation	Random degradation of DNA
Involvement of DNases	DFF40 (CAD), DNaseI, EndoG	DNaseI
Mitochondrial permeability transition pore	May occur late	Early defining event in the mitochondrial necrosis pathway
Loss of inner mitochondrial transmembrane potential	May occur late	Early defining event in the mitochondrial necrosis pathway
Differences		
Membrane	Maintaining of membrane integrity, raising membrane blebs	Loss of membrane integrity, raising non-characteristic membrane blebs
Nuclear morphology	Chromatin condensation and fragmentation of the nucleus	Chromatin condensation, disintegration of chromatin and karyolysis
Cell fate	Ends with fragmentation of cell into apoptotic bodies	Ends with total cell lysis
HMGB1 release	Sequestrated with chromatin into apoptotic bodies	Passive release from nucleus into the cytoplasm
ATP levels	Maintained but may decrease	ATP rapidly depleted
Inflammation	No inflammatory response	Significant inflammatory response
Caspase involvement	Activation of caspase cascade	May occur with Outer Mitochondrial Membrane rapture