Perspective on AMD Pathobiology: A Bioenergetic Crisis in the RPE

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AMD is the leading cause of blindness in developed countries. The dry form of AMD, also known as atrophic AMD, is characterized by the death of RPE and photoreceptors. Currently, there are no treatments for this form of the disease due in part to our incomplete understanding of the mechanism causing AMD. Strong experimental evidence from studies of human donors with AMD supports the emerging hypothesis that defects in RPE mitochondria drive AMD pathology. These studies, using different experimental methods, have shown disrupted RPE mitochondrial architecture and decreased mitochondrial number and mass, altered content of multiple mitochondrial proteins, increased mitochondrial DNA damage that correlates with disease severity, and defects in bioenergetics for primary RPE cultures from AMD donors. Herein, we discuss a model of metabolic uncoupling that alters bioenergetics in the diseased retina and drives AMD pathology. These data provide the rationale for targeting the mitochondria in the RPE as the most efficacious intervention strategy if administered early, before vision loss and cell death.

Keywords: retinal metabolism, retinal pigment epithelium, photoreceptors, mitochondria, human donor tissue
Extensive mitochondrial damage can initiate cell death via the release of mitochondrial proteins (i.e., cytochrome c, apoptosis-inducing factor) and mtDNA through opening of the mitochondrial permeability transition pore.\textsuperscript{16,17} The presence of these mitochondrial proteins and mtDNA in the cytoplasm triggers apoptosis and inflammasome activation, with the latter process leading to necroptosis or pyroptosis.\textsuperscript{18–20} The release of mtDNA into the cytoplasm activates the inflammasome and triggers sterile inflammation.\textsuperscript{16,18} Thus, mitochondria form the central hub for multiple cellular signaling events, including cell death.

**EVIDENCE FOR RPE MITOCHONDRIAL DAMAGE IN AMD**

In the early 2000s, the role for oxidative stress in the etiology of AMD was beginning to emerge.\textsuperscript{21} Data supporting this notion included evidence from human donor tissue that showed increased advanced glycation end products and \( \alpha \)-carboxyethylpyrroles, which are products of protein oxidation, in the retinas of donors with AMD.\textsuperscript{22–31} Oxidative stress can elicit a compensatory response by upregulating expression of proteins that counteract ROS or protect against ROS-induced damage. Data showing elevated levels of multiple antioxidant enzymes and heat shock proteins in RPE from donors with AMD provided indirect evidence for increased oxidative stress.\textsuperscript{3} Positive clinical outcomes from the Age-Related Eye Disease Study also supported a link between oxidative stress and AMD.\textsuperscript{32} This clinical trial sponsored by the National Eye Institute showed supplements of antioxidants plus zinc slowed progression of the disease. Although these studies helped direct the focus to oxidative stress, they did not provide the mechanistic details required to understand the pathogenic changes driving the disease.

During this decade, multiple “discovery-based” approaches, such as proteomics and genomics, were being used to gain insight into disease mechanism. Our early efforts to elucidate the molecular details of AMD used an unbiased proteomics approach to determine how the proteome was altered with AMD progression in both the neural retina and RPE. These analyses used quantitative two-dimensional gel electrophoresis followed by mass spectrometry to identify proteins with altered content. Investigation of the neural retina found 26 proteins exhibited differential content with AMD progression. Most of these proteins were involved in microtubule regulation and transport machinery. This second analysis of mitochondria isolated from RPE showed multiple subunits of the ETC and mitochondrial heat shock proteins, suggesting potential defects in energy production and the process of translocation and refolding of nuclear-encoded proteins that reside in the mitochondria. These early results provided the first indication that the mitochondria in RPE are a potential site of AMD pathology and supported the rationale for performing a more in-depth analysis of the mitochondrial proteome. This second analysis of mitochondria isolated from RPE showed multiple subunits of the ETC and mtHSP70 exhibited decreased content, thereby reconfirming the initial hypothesis that defects in energy production and mitochondrial protein import and refolding occurs with AMD.\textsuperscript{35}

Two additional proteins, mitofilin and mitochondrial translation factor Tu (Tufm), were increased in RPE from donors at an early stage of AMD. Mitofilin is involved in maintaining mitochondrial cristae stability and, therefore, increased mitofilin content may act to combat the destabilizing...
effects of AMD. Consistent with this idea, a study of RPE mitochondrial ultrastructure reported AMD donors had a significantly greater loss of cristae and matrix density compared with their age-matched controls.40 Furthermore, the finding that AMD donors had a significant reduction in the number and area of mitochondria per cell supports the notion that defects in the RPE mitochondria contribute to AMD pathology.

Tufm delivers aminoacylated tRNAs to the mitochondrial ribosome for production of the 13 mitochondrial-encoded proteins. Previous studies showed that overexpression of Tufm rescued mitochondrial phenotypes caused by a MELAS (mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes) tRNA mutation41 and a translation defect resulting in an OXPHOS enzyme deficiency.42 Thus, the dramatic 4-fold upregulation in Tufm content could be a compensatory response in an effort to counter defects in mitochondrial translation that occur early in AMD.

A process that could disrupt mitochondrial translation involves damage to mtDNA in the form of strand breaks and/or addition of DNA adducts. To directly test whether mtDNA damage was increased in macular RPE from AMD donors, damage was measured using a long-extension PCR. This assay is based on the principle that strand breaks or covalent DNA modifications can slow down or block amplification by DNA polymerase. Consequently, the amount of amplified product is proportional to the amount of undamaged endogenous template. Analysis of two separate cohorts that included a total of 53 age-matched controls and 88 donors with AMD showed mtDNA damage correlated with AMD severity.15,39 A more refined analysis of the mitochondrial genome localized the sites of increased damage to regions encoding the 16S and 12S ribosomal RNA and 8 of the 22 tRNAs.43 Defects in these genes could adversely affect translation of the 13 mitochondrial-encoded proteins. Other significantly damaged regions included genes for OXPHOS subunits and the D-loop, potentially leading to reduced ATP production and disruptions in translation and replication.

As previously outlined, mitochondria can also regulate cell death via apoptosis, necroptosis, and pyroptosis. Cell death is particularly relevant to the geographic atrophy observed in advanced dry AMD. The presence of TUNEL staining in the nuclei of photoreceptor and RPE located near the edges of atrophy provides evidence for DNA cleavage and impending cell death. However, the exact mechanism of cell death is not known because DNA cleavage is a shared outcome of apoptosis and necroptosis. Upregulated inflammasome proteins in AMD-affected eyes were observed near drusen and the edges of geographic atrophy.40-42 These data suggest the involvement of either necroptosis or pyroptosis in AMD cell death because both pathways are activated by the inflammasome.19,20 As a caveat to these data, a recent article reporting extensive characterization of inflammasome antibodies called into question the validity of these results.43 However, it is conceivable that the pathogen-associated molecular patterns derived from RPE mitochondria could activate inflammasomes in microglial cells, where the presence of inflammasomes is indisputable.44

In summary, strong evidence from analysis of human donors with AMD supports the emerging hypothesis that defects in RPE mitochondria drives AMD pathology. These studies, using different experimental methods, have shown disrupted RPE mitochondrial architecture and decreased mitochondrial number and mass, altered content of multiple mitochondrial proteins, and increased mtDNA damage that correlates with disease severity. Importantly, damage to the mitochondria would have a significant negative impact on ATP production, leading to a bioenergetic crisis in the RPE.

Rapid postmortem changes that occur in the mitochondria negate the possibility of directly measuring mitochondrial function in human donor tissue. To overcome this limitation, we generated primary cultures of RPE from human donors with and without AMD. These cultures retain many of the prototypic characteristics of RPE in vivo, including cobblestone morphology, the presence of pigment and prototypic RPE proteins, and the ability to phagocytose outer segments.45 To directly test if there were differences in bioenergetics associated with AMD, the bioenergetic profiles of two major energy pathways, glycolysis and mitochondrial OXPHOS, were measured using the Seahorse Extracellular Flux Analyzer. Results showed that in RPE from AMD donors both glycolysis and mitochondrial OXPHOS were significantly decreased.45 In primary cultures from AMD donors, Golestaneh and colleagues46 also found reduced ATP production via OXPHOS, but ATP produced via glycolysis was increased in their experimental system. These results are consistent with the hypothesis that RPE mitochondria are damaged with AMD and, consequently, the ensuing bioenergetic crisis drives AMD pathology.

A Model Explaining How a Bioenergetic Crisis in the RPE Drives AMD Pathology

The work thus far implicates the mitochondria, and overall alterations to bioenergetics in RPE, as a potential mechanism driving AMD pathology. Recent publications by the laboratories of Hurley et al.57 and Philip4 have promoted the idea of metabolic coupling between the RPE and retina. This concept provides a potential explanation for how reduced RPE mitochondrial function could have a global effect on the retina. Based on a series of elegant studies, Kanow and colleagues48 propose that the neural retina and RPE are part of a “metabolic ecosystem,” whereby each cell is co-dependent on the other cell for survival. In this working model (Fig. 2), glucose from the blood is largely unusable by the RPE and is transported to the photoreceptors.48 Photoreceptors use glucose through glycolysis to produce energy and the by-product lactate, which is transported to the RPE where it is used for OXPHOS. An important part of this highly regulated process is the suppression of glycolysis in the RPE by lactate, thereby preserving glucose as an energy source for the photoreceptors.48 Photoreceptors also supply the RPE with a large amount of lipids through daily phagocytosis and digestion of the photoreceptor outer segments. These lipids are substrates for β-oxidation, which produces acetyl CoA, feeding into the TCA and producing β-hydroxybuturate, an alternative energy source for the retina.49 Although other energy sources, such as glutamine,50 proline,51 and glycogen52 are not specifically mentioned in RPE in vivo, they would likely contribute to the flow of energy substrates between the retina and RPE.

AMD disrupts this metabolic ecosystem. As mitochondria are the site of OXPHOS and β-oxidation, damage to this organelle in the RPE would reduce ATP production. RPE would begin to rely on glycolysis to maintain the cell’s energy requirement, thereby reducing the flow of glucose to the photoreceptors. Decreased photoreceptor glycolysis could have multiple effects, including reduced production of lactate for RPE to use as an energy source. Limited suppression of glycolysis due to decreased lactate promotes glucose utilization by the RPE, starving photoreceptors, leading to degeneration and cell death. It has been well documented in AMD that rod death precedes the loss of cones.40,53-54 This observation is relevant to the metabolic co-dependence that cones have with rods. Rods secrete an inactive thioreredoxin, coined rod-derived cone viability factor, which promotes glucose uptake by the
cones and stimulates glycolysis. In the context of changes in RPE metabolism due to AMD-induced mitochondrial damage, the reduced flow of glucose to the photoreceptors coupled with rod death would accelerate the loss of macular cones, a hallmark of advanced AMD.

**MITOCHONDRIA AS A THERAPEUTIC TARGET**

Genetic analysis of AMD patients revealed associations between increased risk for AMD and more than 30 loci involved in multiple pathways, including complement/innate immune system, lipid/cholesterol regulation, cell survival, extracellular matrix remodeling, and angiogenesis. Although this information has been exceptionally useful in formulating specific hypotheses about mechanisms that may be involved in disease development, they imply that a single treatment may not be universally effective. Therefore, we need to develop methods to decipher which aberrant pathway or process is contributing to disease progression in AMD patients, and then use drugs to interrupt or supplement these defects. Additionally, the most efficacious treatment will be one that is administered early in the disease, before retina/RPE cell death and vision loss.

Based on our analysis of RPE mtDNA damage, we found significant mitochondrial damage occurred at an early disease stage preceding when vision loss would occur in AMD patients. This was an exciting finding, as it suggests an early intervention targeting the mitochondria could prevent vision loss in individuals with the greatest mitochondrial damage. With this goal in mind, we used human donor tissue, phenotyped for the presence and severity of AMD and genotyped for AMD risk alleles, to determine if the greatest damage was associated with a specific high-risk genotype. Our analysis found that donors carrying the high-risk allele for the Complement Factor H (CFH) single nucleotide polymorphism (rs1061170) that causes the Y402H mutation had significantly more mtDNA damage compared with donors harboring the CFH wild-type allele. CFH, a component of the innate immune system, prevents inappropriate complement activation thereby keeping inflammation in check. The reduced function of the Y402H variant permits the occurrence of retinal inflammation and tissue damage, promoting invasion of immune cells that release ROS and cytokines. These extracellular signaling molecules upregulate cell pathways (i.e., nuclear factor κB) that increase intracellular oxidative stress in the surrounding tissue. These events could ultimately cause the observed RPE mtDNA damage early in AMD.
This fortuitous discovery linking the CFH high-risk variant with increased mtDNA damage, as well as our earlier result using donors with a mixture of genotypes, was potentially influenced by our sample population from Minnesota, where a high percentage of the ancestry is from Northern Europe. This group has the highest risk for AMD. Notably, approximately 60% of our donors harbored the CFH high-risk allele, whereas in the general population 30% to 50% of all AMD patients carry this risk variant. We postulate that individuals with the CFH risk allele would have the greatest positive response to a mitochondrial-targeted therapy aimed at enhancing or protecting mitochondrial function. Results from this study also provide a roadmap for using donor tissue, coupled with an accessible biomarker (i.e., genotype, clinical phenotype) in patients, as a way to define defective pathways in a specific patient population and then develop therapies targeting the primary defect. This strategy would be one way to move toward “personalized medicine” in treating AMD.

**Bioenergetics, Metabolic Uncoupling, and Critical Unanswered Questions**

One of the preeminent questions in AMD research is “Why the macula?” Specifically, why is there preferential death of macular RPE and photoreceptors when both clinical (peripheral drusen and geographic atrophy) and biochemical (protein changes in peripheral RPE and retina) evidence shows that the detrimental effects of AMD impacts the entire retina? A potential explanation involves the higher bioenergetic demand in the macula and metabolic uncoupling of the retinal ecosystem with AMD. Results from analysis of RPE mtDNA damage showed damage was present throughout the retina of AMD donors, suggesting a global bioenergetic crisis in the RPE that would eventually starve the overlying photoreceptors. The significantly greater loss of macular photoreceptors could be due to the higher density of cones in this region. As discussed previously, rods die first in AMD and cones depend on rods to secrete a neuroprotective factor that improves their glucose uptake. Death of macular rods would have a more significant impact on neighboring cones, as fewer nearby rods are available to support cone metabolism. The higher overall metabolic activity of the macula, combined with death of rods, would contribute to the accelerated loss of both RPE and photoreceptors in the macula with AMD.

We also have observed that although RPE mtDNA damage was increased with AMD, damage to the neural retina was low and did not change with disease progression. This raises the question “Why is mitochondrial damage limited to the RPE?” The answer may involve cell-specific differences in metabolism. RPE rely almost exclusively on mitochondria as an energy source, whereas photoreceptors depend on glycolysis. Recall that a by-product of mitochondrial OxPhos is ROS, which can damage mtDNA. Thus, the lower mtDNA damage observed in the retina is consistent with a reliance on glycolysis rather than OxPhos. The link between the amount of mtDNA mutations and reliance on a specific metabolic pathway was also observed when comparing tumor cells with adjacent non-tumor cells. A lower mutation rate was found in tumor cells that had undergone a shift in metabolism from OxPhos to aerobic glycolysis, a process known as the “Warburg effect.” Hence, the mtDNA mutation frequency in these cells appears to be inversely proportional to the cells’ dependence on glycolysis. Of note, the Warburg effect was first described by Otto Warburg in two tissues, tumors and retinas. However, his reported result from the neural retina was largely ignored until the recent resurgence in studies of retinal metabolism. It is interesting to consider that pathobiology could be precipitated by the contributions of the photoreceptor’s unique metabolism and the metabolic uncoupling that occurs with AMD.

**Summary**

This perspective provides evidence for the hypothesis that a bioenergetics crisis in the RPE underlies the pathobiology of AMD. Although our focus has been on metabolism, the potential ramifications that mitochondrial dysfunction would have on cell signaling, calcium handling, and gene expression that would adversely affect RPE cell function should also be considered. Targeting the mitochondria in the RPE may provide the most efficacious intervention strategy if administered early, before vision loss and cell death.

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