Review

Oxidative stress-induced mitochondrial DNA damage in human retinal pigment epithelial cells: a possible mechanism for RPE aging and age-related macular degeneration

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Abstract

Oxidative stress is believed to contribute to the pathogenesis of many diseases, including age-related macular degeneration (AMD). Although the vision loss of AMD results from photoreceptor damage in the central retina, the initial pathogenesis involves degeneration of RPE cells. Evidence from a variety of studies suggests that RPE cells are susceptible to oxidative damage. Mitochondrial DNA (mtDNA) is particularly prone to oxidative damage compared to nuclear DNA (nDNA). Using the quantitative PCR assay, a powerful tool to measure oxidative DNA damage and repair, we have shown that human RPE cells treated with \( \text{H}_2\text{O}_2 \) or rod outer segments resulted in preferential damage to mtDNA, but not nDNA; and damaged mtDNA is not efficiently repaired, leading to compromised mitochondrial redox function as indicated by the MTT assay. Thus, the susceptibility of mtDNA to oxidative damage in human RPE cells, together with the age-related decrease of cellular anti-oxidant system, provides the rationale for a mitochondria-based model of AMD.

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1. Introduction

The retinal pigment epithelium (RPE) is a monolayer of cuboidal cells located between the photoreceptors of the neurosensory retina and the choroidal capillary bed. It provides many important functions essential to the visual process. By virtue of its location, and presence of tight junctions, RPE cells facilitate selective molecular transport between the choroidal blood and the outer neural retina, thus forming the outer blood-retina barrier. In addition, RPE is responsible for phagocytosis and degradation of shed photoreceptor outer segments. The disruption of these processes has been shown to result in retinal degeneration in experimental animal models (D’Cruz et al., 2000; Gal et al., 2000), and defects in the RPE contribute to initiation and/or progression of age-related macular degeneration (AMD) in humans (Dorey et al., 1989; Green and Enger, 1993).

2. Age-related macular degeneration (AMD)

AMD is a degenerative condition of the central retinal cone-rich zone called the macula. It is a common cause of vision loss in Western countries, and its prevalence is rising with increasingly aged population (Leibowitz et al., 1980; Klein et al., 1992). Currently there is no cure for it and treatment options are limited. AMD is characterized by clinical signs ranging from a few soft drusen and pigmentary changes in the macular RPE with normal visual acuity to large areas of RPE atrophy or choroidal neovascularization with legal blindness. Although the vision loss of AMD results from photoreceptor damage in the central retina, the initial pathogenesis involves degeneration of RPE (Green et al., 1985; Spraul et al., 1996). Progressive RPE dysfunction and death cause a secondary degeneration of rods and cones. This may be due to the close interaction between the RPE and photoreceptor cells in both nutritional and metabolic processes.

While the cause of AMD is not conclusively known, it has been suggested that multiple factors may be involved...
including environmental, nutritional and genetic factors. Among the environmental factors, exposure to sunlight and cigarette smoking have been linked to the risk of AMD development (Cruikshanks et al., 1993; Hammond et al., 1996; Smith et al., 1996; Darzins et al., 1997). Intense illumination or toxic compounds in the tobacco smoke can induce generation of reactive oxygen species (ROS) (Church and Pryor, 1985; Chow et al., 1986; Dorey et al., 1990), thus leading to increased oxidative stress. The nutritional risk factor involves low dietary intake of antioxidants (Seddon and Hennekens, 1994). Increased dietary intake and serum levels of specific antioxidant nutrients may reduce the risk for AMD (Snodderly, 1995).

The linkage between antioxidant micronutrients and AMD was further strengthened by the AREDS clinical trial results which demonstrated a significant reduction in the rate of AMD progression in subjects taking antioxidant and zinc-containing supplements (AREDS group, 2001).

Several studies have suggested that there may be a genetic linkage in AMD epidemiology. A higher prevalence was found between monozygotic twins and among the first-degree relatives with AMD (Klein et al., 1994; Seddon et al., 1997). The association of AMD to genetic factors is further supported by identification of the disease-carrying gene for Stargardt disease, a rare early onset macular dystrophy, which shows some clinical and pathological features with AMD (Allikmets et al., 1997; Briggs et al., 2001). The genetic risk for AMD has been recently estimated as only 23% (Klaver et al., 1998). Thus, the genetic contribution to AMD, although important, appears to be minor compared to environmental factors.

Most of the known risk factors for AMD such as environmental and nutritional factors appear to have oxidative stress as a common denominator (see reviews Winkler et al., 1999; Beatty et al., 2000; Cai et al., 2000). We hypothesize that chronic oxidative stress causes molecular and cellular damage in susceptible RPE cells, which in turn may lead to the pathological and clinical findings seen with AMD. In this review article we provide current information on oxidative stress induced DNA damage and repair in the human RPE cells, with specific emphasis on the mitochondrial (mt) DNA.

3. Mitochondrial susceptibility to oxidative damage

Oxidative stress refers to cell injury caused by ROS such as superoxide anion, hydroxyl radical, singlet oxygen, and H$_2$O$_2$. ROS are produced by a variety of pathways of aerobic metabolism; however, the major source of their production is the mitochondria. Mitochondrial oxidative phosphorylation is a powerful source of ROS with up to 4–5% of the oxygen picking up electrons directly from the flavin dehydrogenases and ubiquinol to generate superoxide radicals (Chance et al., 1979; Richter et al., 1988). Being a major producer of ROS, mitochondria may thus be subjected to direct attack of ROS. Indeed, mitochondrial membrane lipids, proteins, and nucleic acids are all subject to ROS attack. mtDNA is particularly prone to oxidative damage compared to nuclear DNA (nDNA). This is likely due to the fact that mtDNA is located in the matrix, which is in proximity to the ROS-generating respiratory chain; it is not covered by histones and other DNA-associated proteins, thus directly exposed to ROS; furthermore, it is an intronless DNA with high transcription rate, thus providing a high probability of oxidative modification of the coding region; finally, mtDNA repair systems appear to be less efficient (see below). Many studies (Salazar and Van Houten, 1997; Yakes and Van Houten, 1997; Ballinger et al., 1999, 2000; Mandavilli et al., 2000; Jin et al., 2001b; Sawyer et al., 2001) have supported the idea that the mtDNA is more susceptible to damage by ROS than nDNA. Thus, mtDNA damage is a good biomarker of oxidative stress.

Cellular lipids and proteins can also be damaged by ROS, such as lipid peroxidation (Gardner, 1989; Babbs and Steiner, 1990) and protein oxidation (Stadtman, 1992). Lipid peroxidation may stimulate secondary ROS reactions due to electrophiles generated within the mitochondrial membrane. Secondary reactive oxygen reactions may then lead to continued mtDNA damage due to its position on the matrix side of the inner membrane. Since mtDNA encodes proteins involved in electron transport, mtDNA damage will lead to a decrease in mt mRNA and protein synthesis. Loss of these mitochondrial proteins will lead to inhibition of electron transport, the generation of ROS and subsequent more damage to mtDNA, which forms a vicious circle of oxidative damage.

4. Mitochondrial theory of aging

Oxidative damage is believed to contribute to the pathogenesis of many diseases as well as aging. One of the most prevalent theories of aging is the mitochondrial theory: It proposes that oxidative damage to the mitochondria can lead to a spiral of confounding effects, whereby damaged mitochondrial in turn release more ROS, increasing oxidative damage, and leading eventually to dysfunctional or defective mitochondria (Harman, 1981). Because mitochondrial respiration is essential for the production of ATP, damage to mitochondria as a result of oxidative stress could result in reduced energy production and compromised cell function. Over time, deficits in cellular function caused by this cycle of oxidative damage could become amplified and contribute to age-related declines in physiological function.

Damage to mtDNA probably has more relevance to the mitochondrial theory of aging than damage to lipid or protein because mtDNA damage can be propagated as mitochondria and cells divide, thus allowing the physiological consequences of the damage to be amplified. In addition, damage to mtDNA could be potentially more
important than deletions in nDNA, because the entire mitochondrial genome codes for genes that are expressed while nDNA contains a large amount of non-transcribed sequences. Also, mtDNA, unlike nDNA, is continuously replicated, even in terminally differentiated cells, such as neurons and cardiomyocytes; hence, somatic mtDNA damage potentially causes more adverse effects on cellular functions than does somatic nDNA damage.

5. RPE susceptibility to oxidative stress

Evidence from a variety of studies suggests that RPE cells are susceptible to oxidative damage. Anatomically, RPE is located between the sensory retina and choroid. This close location exposes RPE cells to a highly oxidative environment due to high oxygen partial pressure from the underlying choriocapillaries. Physiologically, RPE cells phagocytose and digest photoreceptor outer segments. This unique phagocytic function of RPE provides an additional oxidative burden since the shed outer segments are extremely rich in polyunsaturated fatty acids (PUFA) having perhaps one of the highest concentrations of PUFAs of all membrane systems (Snodderly, 1995). Oxidation of PUFA initiates a chain reaction producing an abundance of ROS, including lipid aldehyde radicals (Esterbauer et al., 1991; Srivastava et al., 1995). In addition, the process of RPE phagocytosis is itself an oxidative stress and results in the generation of endogenous ROS (Tate et al., 1991; Jin et al., 2001b). Furthermore, RPE cells contain an abundance of photosensitizers, and exposure to intense visible lights induces generation of ROS (Gaillard et al., 1995; Rozanowska et al., 1995). To cope with these toxic oxygen intermediates, the RPE has evolved effective defenses against oxidative damage. It is particularly rich in anti-oxidants such as vitamin E, superoxide dismutase, catalase, glutathione-S-transferases, glutathione, and ascorbate (Newsome et al., 1990; Beatty et al., 2001). However, with increasing age, the RPE anti-oxidative capability appears to be reduced. For example, catalase activity and hemeoxygenase-1 level decrease in aging RPE cells (Liles et al., 1991; Tate et al., 1993). Thus, it is likely that aging RPE cells may be more susceptible to oxidative damage. Oxidative damage to lipids and proteins has been well studied in RPE cells (Akeo et al., 1996; Ueda and Armstrong, 1996; Kayatz et al., 1999; Wu and Rao, 1999; Tyni et al., 2002); however, little attention has been paid to oxidative damage to the mtDNA.

6. Quantitative measurement of DNA damage

Identification of oxidative lesions in nuclear and mtDNA has been performed by various techniques: HPLC-electrochemical detection of 8-oxoguanine, an oxidatively modified guanine base which accumulates more in mtDNA than in nuclear DNA (Beckman and Ames, 1996), Southern analysis and ligation-mediated polymerase chain reaction (PCR). One disadvantage regarding these techniques is the requirement for large quantities of DNA, which can be limiting when specific tissue or primary cell cultures are used. The techniques also require isolation of the mitochondria before DNA purification, which provides additional opportunity for oxidation to occur during sample preparation. To this end, a novel gene-specific quantitative PCR (QPCR) assay has been developed to measure oxidative DNA damage and repair (Yakes and Van Houten, 1997). Both inactively and actively transcribed genes (such as β-globin and β-polymerase) were chosen for detection of nuclear DNA damage. A 16-2-kb fragment from the mitochondrial genome was amplified for detection of mtDNA damage. Oxidative DNA damage includes single and double-strand breaks, abasic sites, and base damages (Demple and Harrison, 1994). The damage measured by the QPCR assay includes both strand breaks and base modification.

This gene-specific assay technique works on the premise that a lesion on the DNA template will block a thermostable polymerase and result in a decreased amplification of the target sequence (Yakes and Van Houten, 1997). Therefore, only those DNA templates that do not contain polymerase blocking lesions will be amplified. DNA lesions, such as strand break, base modification and apurinic site, are all capable of blocking the progression of the polymerase. Thus, quantification of DNA lesions per genomic fragment is inversely proportional to the resultant PCR product. The current detection limits are 1–2 lesions/10⁵ nucleotides with 5–15 ng mammalian DNA (equivalent to approximately 1000–3000 cells). Because differences in QPCR amplification may at times be related to template copy number differences or simply due to the DNA quality unrelated to in vivo or in vitro mediated damage, a quantitative amplification of a small region is performed as a quality control. Small target regions (i.e. ~ 200 bp) in the DNA are unlikely to suffer any insult, and, thus, can serve as indicators of relative copy number and PCR quality of the genomic extract. We have adopted this technique to study oxidative damage in human RPE cells.

7. Oxidative stress and mtDNA damage in RPE

To study oxidative stress in RPE cells, most studies have used mammalian RPE cells in an in vitro culture system, either primary or transformed cells. One of the drawbacks using primary RPE cells is that they provide a limited number of cells and may lack cellular consistency due to the variability associated with different donor sources. Transformed RPE cells overcome these problems while maintaining morphological and functional characteristics of primary cells. They possess epithelioid morphology, presence of polarity, and the ability of phagocytotize rod outer
segments. Additional studies indicate that these cells express and respond to oxidative stress (H$_2$O$_2$, media starvation, etc.) in a fashion similar to that primary cultured human RPE cells (Sippy et al., 1995). Several approaches have been used to induce oxidative stress of RPE cells, including exposure to chemical oxidants (H$_2$O$_2$, t-butyl hydroperoxide), hyperoxia, feeding with rod outer segments or lipofuscin, and photol illumination.

We chose to use the SV40 transformed fetal human RPE cells to study oxidative stress. RPE cells were exposed to various concentrations (50–200 μM) of H$_2$O$_2$ for different time periods (0–60 min), and mt and nDNA damages were assessed by QPCR. We found mtDNA was significantly damaged by all concentrations of H$_2$O$_2$ (Ballinger et al., 1999). The time course analysis showed that significant damage to mtDNA was detectable within 15 min of exposure. By contrast, both the transcriptionally active and inactive nuclear genes (beta-globin gene cluster, hprt, and beta-polymerase genes) were resistant after a 15-min exposure of H$_2$O$_2$ (Ballinger et al., 1999). The sustained mtDNA damage led to compromised mitochondrial redox function as indicated by MTT assay. These data suggest that mtDNA repair devices in the RPE cells are largely insufficient to overcome extensive DNA damage. What factors could contribute this persistent mtDNA damage? It is possible that repair enzymes in the mitochondria may be inactivated by oxidative damage. This is supported by study showing that nitric oxide inhibited bacterial repair enzyme glycosylase (Laval et al., 1997). In addition, it is likely due to the vicious cycle of ROS production and damage as mentioned earlier, in which a damaged mitochondrion leads to increased ROS production and more mtDNA damage.

Recent evidence suggests that β-polymerase may be induced in response to oxidative stress and thereby protect the DNA from ROS damage (Chen et al., 1998). To see whether BER enzymes are increased after oxidative stress, the levels of DNA polymerase-β and AP endonuclease (APE) were measured by Western blot after RPE cells were treated to H$_2$O$_2$, or rod outer segments. We found that DNA polymerase-β, but not APE, was markedly increased after H$_2$O$_2$ exposure (Godley et al., unpublished data). Interestingly, both enzymes were significantly increased following rod outer segment treatment (Jin et al., 2001b). It is unclear why APE expression is increased with outer segment treatment but not H$_2$O$_2$. Collectively, these data suggest that oxidative stress not only induces mtDNA damage but also activates DNA repair mechanisms in the RPE cells. We predict that exploration of mtDNA repair systems is going to be an important area of research in the coming years. This is particularly true for human RPE cells undergoing aging processes. It may be of interest to study mitochondrial repair capacity in aging human RPE cells and in RPE from eye donors with AMD.

8. Oxidative stress and mtDNA repair in RPE

Because mtDNA is subjected to oxidative damage, it seems that mitochondria would need efficient DNA repair mechanisms to maintain its integrity. The repair of mtDNA has been investigated less extensively than nDNA repair because pyrimidine dimers produced by UV-irradiation are not repaired in mitochondria (Clayton et al., 1974) and this led to the general notion that there is no DNA repair systems in mitochondria. However, recent studies have demonstrated that DNA can be repaired in mitochondria, and base excision repair (BER) is the main repair pathway in mitochondria of mammalian cells (see reviews, Croteau and Bohr, 1997; Mandavilli et al., 2002), although there is no direct evidence that the nucleotide excision repair pathway, which removes UV-induced lesions and other bulky lesions in the nucleus, exists in mitochondria. Several BER enzymes, including DNA glycosylase and AP endonuclease, have been isolated from mammalian mitochondria. The BER pathway is initiated by the action of DNA glycosylase, which removes the aberrant base by cleaving the glycosylic bond, thereby leaving a non-coding abasic site (AP site). The AP site is then recognized and cleaved by an AP endonuclease, which forms a one-nucleotide gap. Finally, the gap is filled by DNA polymerase and the nick is sealed by DNA ligase.

It has been demonstrated that the capacity of BER pathway in mitochondria decreases with age (Cortopassi and Wong, 1999). mtDNA repair in human RPE cells has not been studied prior to our investigation.

As mentioned earlier, we have shown that human RPE cells treated with H$_2$O$_2$ resulted in preferential damage to mtDNA, but not nuclear DNA (Ballinger et al., 1999). In the same study, the rates of mtDNA repair were examined and it was found that the mtDNA sustained only limited repair after a 15-min exposure of H$_2$O$_2$ (200 μM). A longer exposure resulted in persistent mtDNA damage, which was not repaired at all within 3 hr. This was also observed in the transformed human fibroblast cells (Yakes and Van Houten, 1997). The sustained mtDNA damage led to compromised mitochondrial redox function as indicated by MTT assay. These data suggest that mtDNA repair devices in the RPE cells are largely insufficient to overcome extensive DNA damage. What factors could contribute this persistent mtDNA damage? It is possible that repair enzymes in the mitochondria may be inactivated by oxidative damage. This is supported by study showing that nitric oxide inhibited bacterial repair enzyme glycosylase (Laval et al., 1997). In addition, it is likely due to the vicious cycle of ROS production and damage as mentioned earlier, in which a damaged mitochondrion leads to increased ROS production and more mtDNA damage.
9. Oxidative stress and RPE apoptosis

The initial loss of RPE cells in AMD may result from apoptosis (Ishibashi et al., 1986), although the number of apoptotic RPE cells during the early phase could be too low to be detected by available techniques. In surgically excised choriald neovascular membrane from AMD patients and AMD donor eyes, apoptotic RPE cells were readily identified (Hinton et al., 1998; Dunaief et al., 2002). It is hypothesized that oxidative-stress-induced mitochondrial dysfunction plays a pivotal role in the series of molecular events culminating in apoptotic cell death. Our studies have shown that mitochondrial dysfunction is correlated with mtDNA damage (Ballinger et al., 1999). Because persistent mtDNA damage might be an early indicator of a decline of mitochondrial function, we sought to correlate mtDNA damage with mitochondrial function and apoptotic processes. We have found exposure of RPE cells to H$_2$O$_2$ significantly decreased mitochondrial respiratory function as measured by MTT assay and increased expression of pro-apoptotic proteins (p53 and p21 and caspase-3) (Jin et al., 2001a). Also, the expression of anti-apoptotic protein bcl-2 in H$_2$O$_2$-treated RPE cells was diminished while over-expression of this protein reduced cell apoptosis (Godley et al., 2002). Hence, exposure of RPE cells to the concentrations of H$_2$O$_2$ that cause mtDNA damage also promotes apoptosis. This is consistent with findings that exposure of RPE cells to other oxidative stress inducers such as $\gamma$-butyldihydroperoxide, light irradiation and lipofuscin can trigger RPE apoptosis (Cai et al., 1999; Sparrow et al., 2000; Sparrow and Cai, 2001). Together, these data support the notion that oxidative stress induces mitochondrial damage and dysfunction which may in turn trigger apoptosis in RPE cells.

10. Correlation of mtDNA damage with AMD development

The susceptibility of mitochondrial DNA to oxidative damage in the human RPE cells, together with the age-related decrease of cellular anti-oxidant system, provides the rationale for a mitochondria-based model of AMD as summarized in Fig. 1. RPE cells generate ROS through cellular oxidative phosphorylation and phagocytosis. Generation of ROS preferentially damages mtDNA. Consequently, chronic ROS production over several decades leads to constant, steady damage to the mitochondrial machinery and its DNA. Lack of efficient mtDNA repair accelerates accumulation of damaged mtDNA. As damage accumulates, mitochondrial redox function decreases, leading to a vicious cycle of ROS production and mtDNA damage. As a result, mitochondrial function declines, which results in diminished energy production. When the level of energy production drops below the threshold so that mitochondria lose their ability to maintain the membrane potential.

Subsequently, cytochrome c is released into cytoplasm, which initiates apoptosis through the activation of caspases, a possible early pathological event of AMD.

11. Future directions

Most of the current model systems to study RPE oxidative stress used the acute and high dose of either chemical treatment or light exposure. The extrapolation of these data to explain AMD, a chronic, variously progressive disease, must be tentative since the dose of oxidant used in these acute studies might not be relevant to the in vivo condition. It is likely that oxidative stress in vivo is chronic and below levels that cause RPE cell death. Therefore, it would be useful to establish a chronic oxidative stress model system of RPE to simulate the in vivo situation. Recently, Honda et al. (2001) have developed a model of mild and chronic oxidative stress using hyperoxia for RPE cells in vitro. In contrast to the acute models, this model does not affect cell viability while still inducing ROS production.

Another concern is that most studies used RPE cell lines to study oxidative stress. Although RPE cell lines offer some advantages as described earlier, they are transformed and may respond differently to oxidative challenge compared to primary RPE and in vivo RPE. Thus, it would be more appropriate to use freshly isolated or primarily cultured RPE in the future studies. Since the amount of DNA obtained from each donor eye is very limited, QPCR assay may be
particularly useful due to its sensitivity and ability to simultaneously measure mt and nuclear DNA damage. Using QPCR assay, one can examine the formation of oxidative mtDNA damage and repair kinetics in RPE cells from healthy aged human eye donors as well as early AMD eye donors. This will determine whether RPE cells have more baseline mtDNA damage in AMD eye donors compared to healthy age-matched eye donors, and whether RPE cells from these AMD eyes are more susceptible to ROS-mediated mtDNA damage and whether their repair is less efficient.

Finally, there is a need to define the critical molecular events involved in oxidative stress induced RPE cell death. Accordingly, it would be interesting to define and compare differential gene expression in human RPE cells exposed to oxidative stress as well as in normal and AMD-affected human RPE cells by using microarray technology. The response of these RPE cells to oxidative stress may help reveal the mechanisms and pathways that are involved in the development of AMD.

References


