

# Oxidative Damage and Protection of the RPE

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**Abstract**—This review provides a model for the role of oxidative stress in the etiology of age-related macular degeneration (AMD). Epidemiological studies of diet, environmental and behavioral risk factors suggest that oxidative stress is a contributing factor of AMD. Pathological studies indicate that damage to the retinal pigment epithelium (RPE) is an early event in AMD. In vitro studies show that oxidant treated RPE cells undergo apoptosis, a possible mechanism by which RPE cells are lost during early phase of AMD. The main target of oxidative injury seems to be mitochondria, an organelle known to accumulate genomic damages in other postmitotic tissues during aging. The thiol antioxidant GSH and its amino acid precursors protect RPE cells from oxidant-induced apoptosis. Similar protection occurs with dietary enzyme inducers which increase GSH synthesis. These results indicate that therapeutic or nutritional intervention to enhance the GSH antioxidant capacity of RPE may provide an effective way to prevent or treat AMD. © 2000 Elsevier Science Ltd. All rights reserved

## 1. INTRODUCTION

The percentage of aged people in developed countries has increased steadily during the past 50 years. (Harman, 1998). In 1992, the average

life expectancy of men in the United States was 73.2 years, while women lived even longer to an average of 79.7 years (National Center for Health Statistics, 1993). However, despite improved living conditions, aged people still face the threats of many chronic diseases which can greatly affect the quality of their life. One such disease is age-related macular degeneration (AMD).

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AMD is an age-related, progressive degeneration of photoreceptors and their underlying retinal pigment epithelium (RPE) in the macular area of the retina (Klein and Klein, 1982; National Advisory Eye Council, 1982). Because the macula is responsible for high resolution central and color vision, AMD leads to severe central vision impairment and even permanent blindness.

The incidence of AMD has been investigated by several large epidemiological studies. According to the Framingham Eye Study, almost 11% of people aged 65–74 have varying degrees of AMD, and the incidence increases to 28% between ages of 75 and 85 years (Leibowitz *et al.*, 1980). Results from the Beaver Dam Eye Study showed the incidence of AMD was 11% and 30% in age groups 65–74 and beyond 75, respectively (Klein *et al.*, 1992). Two percent of people aged 75–84 suffer from legal blindness, and most of these cases are attributed to AMD (Klein *et al.*, 1992).

Despite the high incidence and severity of vision impairment, only a limited percentage of AMD patients are amenable to treatment (Ciulla *et al.*, 1998). At present, there is no available effective treatment for the non-exudative or ‘dry’ form of AMD that occurs early in disease progression. Patients who are more severely affected with choroidal neovascularization can benefit from laser photocoagulation treatment in cases where the blood vessels have well-defined boundaries (Macular Photocoagulation Study Group, 1991). However, there is a high recurrence rate after laser treatment and it leaves permanent damage to the retina. (Macular Photocoagulation Study Group, 1994). Other alternative treatments, such as surgical macular relocation (Machemer and Steinhorst, 1993), photodynamic therapy, submacular surgery, radiation and pharmacological interventions (Ciulla *et al.*, 1998), are still at the pretrial or early trial stages. Although these approaches are promising, elucidation of the mechanism responsible for AMD would enhance the existing treatments and potentially provide the basis for design of new strategies to either treat or prevent this high incidence disease. In this review, we discuss the available data that support the involvement of oxidative

stress as an important contributing factor in the development of AMD and describe a potential therapeutic strategy which utilizes a GSH synthesis inducer to elevate the antioxidant function of RPE.

## 2. AMD IS A MULTIFACTORIAL DISEASE OF AGING

The early pathological changes of AMD often start with drusen formation in the macular area (Bressler *et al.*, 1990; Green and Key, 1977; Sarks, 1980). Small, hard drusen with well-defined edges are present in more than 90% of eyes from old people and are usually not considered to be pathological. Larger, soft drusen (>63  $\mu\text{m}$  in diameter) are diagnosed as early AMD (Bird *et al.*, 1995; Curcio and Millican, 1999). Large drusen can merge, become confluent and lead to geographic atrophy of the central retina. At the microscopic level, an aggregation of hyaline materials between the Bruch’s membrane and the RPE can usually be detected. The thickness and continuity of the basal laminar deposits are found to be significantly higher in AMD eyes (Spraul *et al.*, 1996).

Although the vision loss results from photoreceptor damage in the central retina, the initial pathogenesis of AMD involves the degeneration of RPE (Green and Key, 1977; Green *et al.*, 1985; Spraul *et al.*, 1996; Zarbin, 1998). Because of the close interaction between RPE and photoreceptors in both nutritional and metabolic aspects (Marmorstein *et al.*, 1998), the progressive RPE dysfunction causes a secondary degeneration of rods and cones. The genetic and biochemical mechanisms responsible for RPE degeneration in AMD have not been determined. Most likely, multiple factors are involved (Snodderly, 1995). These can include environmental, nutritional and behavioral factors as well as different susceptibilities to these external factors based upon different individual’s genetic background.

The higher prevalence of AMD found between monozygotic twins (Klein *et al.*, 1994; Melrose *et al.*, 1985; Meyers and Zachary, 1988) and among first-degree relatives with AMD (Seddon *et al.*,

1997) suggests the existence of genetic factors in predisposing and/or developing the disease. Relatives of exudative AMD patients have a much higher likelihood to develop AMD. Compared to the relatives of control probands, the odds ratio is 3.1 (Seddon *et al.*, 1997). However, environmental factors, dietary intake and smoking habits, could also be shared among family members in addition to the inherited genetic information. If any of these external factors are indeed risk factors of AMD, they can also contribute to the familial aggregation (Seddon *et al.*, 1997).

Several genes have been associated with inherited retinal dystrophies that have similar clinical manifestations to AMD. These include the gene encoding the tissue inhibitor of metalloproteinase-3 (TIMP3) (Ma *et al.*, 1998), the Best gene which is associated with Best macular dystrophy (Pertukhin *et al.*, 1998) and the ABCR gene which is mutated in Stargardt's macular dystrophy (Allikmets *et al.*, 1997a,b) and retinitis pigmentosa (Martinez-Mir *et al.*, 1998).

The ABCR gene is of particular interest because in one study the mutations of the gene were detected in 16% of a cohort of AMD patients, especially those with geographic atrophy (Allikmets *et al.*, 1997a, b). The gene encodes a transmembrane protein which typically has two ATP-binding cassettes (Azarian and Travis, 1997). The function of the protein is unknown but may involve a transport protein for retinoids (Azarian and Travis, 1997; Sun *et al.*, 1999). However, while the common mutations of the ABCR gene in AMD patients suggest a causal association, other subsequent studies showed that the allelic variation rate of ABCR gene is high even in control non-AMD individuals. Among 96 control subjects, Stone *et al.* (1998) found that 26 had non-conservative mutations of the ABCR gene. This rate was similar to that of the AMD patients, where 57 out of 182 patients had alterations of the gene (Stone *et al.*, 1998). Therefore, the detected mutations may simply reflect polymorphisms or mutations found in normal healthy people; whether these mutations impact AMD remains to be determined (Van Driel *et al.*, 1998).

Without a well-defined function, genetic epide-

miological studies cannot securely attribute the etiology of a complicated chronic disease like AMD to mutations of single genes. Like other age-related degenerative diseases, the incidence of AMD rises exponentially with age. In principle, an inherited mutation would be expected to show its abnormal phenotype as soon as after birth, and the functional abnormality should correlate linearly with age rather than exponentially. Therefore, it remains difficult to explain why inherited genetic mutations would affect the function of RPE cells only during the last third of an individual's life.

Several possible mechanisms could explain this characteristic. For instance, mutations in genes that affect the fidelity of replication can result in accumulation of somatic mutations with age in populations of cells that continue to divide. Such a mechanism has been suggested for Werner syndrome which is associated with loss-of-function mutations of WRN gene (Yu *et al.*, 1996). The WRN gene encodes a DNA helicase and mutations of this gene cause rapid shortening of telomeres (Schulz *et al.*, 1996) and high rates of somatic mutations (Fukuchi *et al.*, 1989), which suggest that mutations in a replication factor can result in decreased replication fidelity. However, because the RPE appears to have limited mitosis, other mechanisms may be more likely for AMD. One of these would be mutation in a gene affecting DNA repair. Because damage to the nuclear and mitochondrial genomes is ongoing throughout life, a defect of DNA repair could allow rapid accumulation of damage that ultimately results in manifestation of an accelerated aging process. Such a mechanism has been associated with Cockayne syndrome, in which both the nucleotide excision repair and transcription-coupled repair are deficient (Bohr *et al.*, 1998; Friedberg, 1996).

Alternatively, accumulation of subtle mutations affecting any critical system could decrease cell function to approach a threshold for development of the disease so that any further loss of function, especially those damages caused by environmental agents, would cause an earlier onset of disease with an exponential character in aging individuals. This concept is illustrated in Fig. 1, which is a modification of the

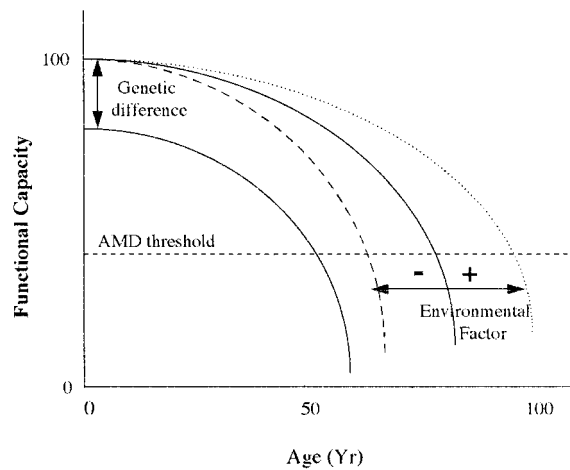


Fig. 1. Schematic time course for the development of age-related retinopathy. AMD is likely a disease resulted from progressive damages to the retina and RPE throughout life and is observed only when function drops below a critical threshold (—). Inherited difference can affect the age of onset without impairment early in life because of reserve functional capacity. However, an individual exposed to certain environmental factors such as oxidative stress develops an accelerated course (- - -) and appropriate intervention may protect against damage and delay the onset (. . .).

threshold model for mitochondrial myopathies proposed by Wallace (1992). In this model, functional capacity in normal individuals greatly exceeds the level at which disease becomes apparent. With aging and accumulation of genetic damages, functional capacity decreases until the disease threshold is achieved. Differences in age of onset can occur because of genetic differences that allow individuals to reach the threshold at an earlier age. Regardless of the genetic background, age of onset can be accelerated by negative risk factors and delayed by positive risk factors. Among the environmental factors that could affect such processes, genomic damage associated with oxidative stress appears to be an important contributor to age-related pathologies.

### 3. AGING IS ASSOCIATED WITH OXIDATION

Under aerobic conditions, reactive oxygen species (ROS) are generated at a very high rate. An individual consumes over 250 g of oxygen

daily, and 1–5% of it is thought to be converted into ROS (Chance *et al.*, 1979). There are a variety of types of ROS, including radicals, such as superoxide ( $O_2^-$ ) and hydroxyl ion ( $OH^\bullet$ ), and non-radicals, such as hydrogen peroxide ( $H_2O_2$ ) and singlet oxygen ( $^1O_2$ ). The main sources of intracellular ROS generation are the mitochondrial electron transport chain, microsomal electron transport chains, NAD(P)H oxidases and, particularly for RPE cells, lipid peroxidation from phagocytosed rod outer segments.

Most of the ROS are byproducts of normal physiological processes and are eliminated immediately by antioxidant systems. The antioxidant enzymes, such as superoxide dismutase, GSH peroxidase and catalase, form the primary defense. Smaller antioxidant molecules, such as vitamin C, vitamin E, ubiquinol and carotenoids, back up the enzymatic systems as direct radical scavengers. Carotenoids (Snodderly, 1995), such as lutein and zeaxanthin, are components of the macular pigments (Hammond *et al.*, 1997; Landrum *et al.*, 1997). They quench the reactive singlet oxygen (Edge *et al.*, 1997; Fukuzawa *et al.*, 1998) and also form an optical filter to cut off the most damaging blue light before reaching the sensory neurons (Frank, 1996; Schalch, 1992). During aging and pathological conditions, the balance between the ROS generation and the ROS clearance can be disturbed and result in oxidative damage to macromolecules (Ames *et al.*, 1993; Harman 1998).

Strong evidence supporting the role of oxidative stress in aging has been obtained from studies on lower animals such as fruit flies and nematodes. Simultaneously overexpressing Cu/Zn-superoxide dismutase and catalase increased the *Drosophila*'s life span by 34% (Orr and Sohal, 1994). Noticeably, these flies had nearly normal phenotype, which suggested the increased life was not due to lack of movement or low metabolic rate. Similarly, increased levels of SOD and catalase have been also found in *C. elegans* age-1 mutants which have life-expectancies twice as long as wild type *C. elegans* (Vanfleteren and De Vreese, 1996; Larsen, 1993).

However, the mechanisms controlling aging are much more complicated in mammals. Whether the aging process is genetically pro-

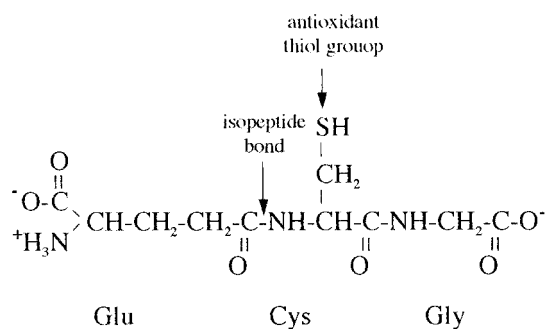


Fig. 2. Molecular structure of the reduced form glutathione. It is tripeptide of glutamate, cysteine and glycine. The  $-SH$  group of the cysteine is used as an electron donor in antioxidant reactions. This group also enables it to react with electrophiles, such as 4-hydroxynonenal produced during lipid peroxidation. The  $\gamma$ -glutamyl peptide bond makes glutathione resistant to peptidases.

grammed and involves regulation by specific genes, such as the newly identified WRN gene (Yu *et al.*, 1996), remains uncertain (Johnson *et al.*, 1999). Overexpression of Cu/Zn-SOD in mice leads to premature aging and neuropathological abnormalities that are observed in Down's syndrome patients (Avraham *et al.*, 1988). The only extensively studied experimental model leading to prolonged life in mammals involves restriction of calorie intake (Barzilai and Gupta, 1999; Masoro 1996). Because both the metabolic rate and the ROS production are decreased in this model, the effects cannot be attributed solely to oxidative stress even though this is an attractive hypothesis.

Age-related decreases in the function of antioxidant systems have been reported by many studies. One method to quantitate changes in antioxidants during aging is to measure their blood plasma concentrations. We measured one of the major water soluble antioxidants, glutathione (GSH), in plasma from volunteers with different ages. GSH is a tripeptide,  $\gamma$ -Glu-Cys-Gly (Fig. 2), and serves both as an important substrate for the enzymatic antioxidant systems such as glutathione peroxidase and as a direct antioxidant (Jones *et al.*, 1995; Reed, 1990). The isopeptide bond of the  $\gamma$ -Glu-Cys linkage prevents GSH from being degraded by most proteases and peptidases. In addition, this linkage stabilizes the thiol group which is relatively reac-

tive in free cysteine. Consequently, very high concentration of GSH can be maintained in cells; whereas, similar concentration of cysteine would be toxic. The stabilized thiol of GSH is used as an antioxidant. In such reactions, the thiol group is oxidized to form a disulfide bond between two molecules of GSH and the oxidized GSH is therefore designated as GSSG. GSSG can be reduced back to GSH by the NADPH dependent GSSG reductase.

In a recent study with more than 100 participants, we found that the plasma GSH concentration decreased with age. In individuals younger than 60, the geometric mean of measured GSH was  $1.89 \pm 0.24 \mu\text{M}$ , while in individuals older than 60, GSH decreased to  $1.32 \pm 0.13 \mu\text{M}$  (Samiec *et al.*, 1998). These data are consistent with earlier studies that showed an age-related decrease in plasma GSH (Kretzscharm and Muller, 1993; Lang *et al.*, 1992). In parallel to the GSH decrease, we also found that the estimated plasma GSSG concentration increased from  $0.015 \mu\text{M}$  in younger individuals to  $0.27 \mu\text{M}$  in older individuals. The increase in GSSG indicates that there is both a loss in blood plasma GSH and also an oxidation of the circulating thiol-disulfide pool.

Oxidation of GSH to GSSG is a two electron reaction. Therefore, we used the Nernst equation  $E_h = E_0 + RT/F \ln([GSSG]/[GSH]^2)$  to estimate the redox potential for the GSH/GSSG redox couple in the plasma, where  $R$  is the gas constant,  $T$  is the absolute temperature and  $F$  is Faradays constant.  $E_0$  was taken as  $-240 \text{ mV}$  (Rost and Rapoport, 1964). The  $E_h$  was found to be 45 mV more oxidized in the subjects older than 60 (Samiec *et al.*, 1998). Thus, the data suggest that a substantial oxidation of the thiol-disulfide pool occurs in the blood plasma as a function of age.

This conclusion is supported by studies of other plasma antioxidants, such as vitamin C (Rikans and Moore, 1988) and vitamin E (Vandewoude and Vandewoude, 1987), which have also been found to decrease with age. In addition, the plasma thiobarbituric acid reacting substances (TBARS) increase with age, indicating increased lipid peroxidation (Coudray *et al.*, 1997). Together with the oxidation of the gluta-

thione pool, these observations suggest a generalized shift of the plasma redox state to be more oxidized during aging.

In accordance with the decrease of antioxidants with age, increased ROS induced-damage has been found in a variety of tissues, especially postmitotic tissues, such as brain, skeletal muscle, heart and liver (Shigenaga *et al.*, 1994). The protein carbonyl contents increase in rat brain and liver (Tian *et al.*, 1998). The protein tyrosine oxidation product, O,O'-dityrosine, increases with age in mouse skeletal and cardiac muscles (Leeuwenbrugh *et al.*, 1997). As a marker of DNA oxidative damage, 8-hydroxy-2-deoxyguanosine increases with age in brain, heart, liver and kidney in rats (Kaneko *et al.*, 1997) and increases in biopsy samples from human skeletal muscle (Mecocci *et al.*, 1999). Most of these changes can be prevented or attenuated by caloric restriction, suggesting that they occur as a consequence of metabolism of energy yielding food stuffs, a process which occurs principally by the oxidative system in the mitochondria. Age-related oxidative damage to specific organelles, especially the mitochondria, has been extensively studied (Wallace *et al.*, 1998). A positive correlation between mitochondrial DNA damage and the altered function has been established ((Brierley *et al.*, 1998; Kopsidas *et al.*, 1998). As discussed below, the accumulation of mitochondrial damage may contribute to the loss of cells during the aging process.

#### 4. RPE AGING AND OXIDATIVE STRESS

Similar to other parts of the body, RPE is also subjected to age-related oxidation and the resultant oxidative stress. ROS generated from phagocytosis and lipid peroxidation (Tate *et al.*, 1995), intense illumination from focal light (Dorey *et al.*, 1990), and together with high oxygen tension in the macular area (Alder and Cringle, 1985) all suggest that oxidative stress may be particularly significant in the RPE. In addition, the unique phagocytic function of RPE provides an additional oxidative burden (Kennedy *et al.*, 1995). The turnover rate of photoreceptors is high. With constant shedding of the outer segments

and synthesis of new membranes, a photoreceptor can achieve total renewal within 10 days. The shed membranes have the highest concentration of polyunsaturated fatty acids (PUFAs) of any human tissue and are promptly phagocytosed by RPE. Peroxidation of these lipids could induce damage in RPE. The lesions induced by oxidative injury may accumulate and trigger affected RPE cells to undergo apoptosis. Undigested materials from dead RPE cells could be released and deposited into the inner layer of Bruch's membrane and drusen formation (Ishibashi *et al.*, 1986).

Along with the physiological oxidative stress, environmental factors, such as cigarette smoking (Chan, 1998) and intense light exposure (Bressler and Bressler, 1995; Cruickshanks *et al.*, 1993) also increase the risk for RPE degeneration. Smokers have been found to have lower plasma vitamin C and carotenoids (Chow *et al.*, 1986). Their red blood cells have increased tendency to peroxidize *in vitro* (Duthie *et al.*, 1991). Toxic compounds in the tobacco smoke, including carbon monoxide and nicotine, can induce both hypoxia and ROS generation (Church and Pryor, 1985). In a prospective study, heavy cigarette smokers had 2.4 times higher risk of AMD (Seddon *et al.*, 1996). Similar data have been reported with other large scale case-control and cross-sectional studies (Chan, 1998).

The antioxidant capacity of RPE decreases with age. When assayed using human donor eyes, catalase activity in both macular and peripheral RPE was found to be negatively correlated with donor age (Liles *et al.*, 1991) while the superoxide dismutase activity remained unchanged. Illumination of isolated RPE cells with blue light induced an increase of oxygen consumption and hydrogen peroxide production that was associated with age of the individual (Rozañowska *et al.*, 1995). Retinal cells from older individuals have higher accumulation of age-related pigment, lipofuscin, than most other tissues in the body (Katz *et al.*, 1984). The function of lipofuscin is still controversial but has been suggested to decrease the lysosomal activity of RPE (Holz *et al.*, 1999) and also to pass the radiation energy into the cytoplasm (Brunk *et al.*, 1995; Sundelin *et al.*, 1998; Wihlmark *et al.*, 1997). Either of

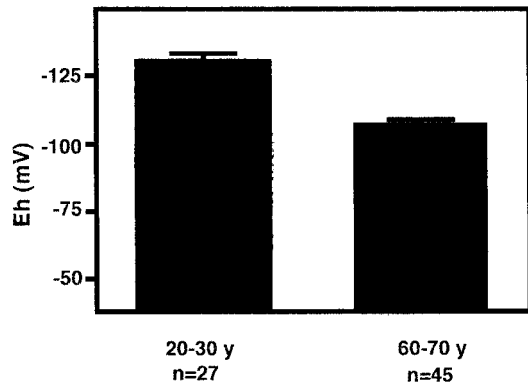


Fig. 3. Age-related oxidation of plasma GSH redox potential. The estimated values of  $E_h$  in younger and older groups are  $129 \pm 1.2$  mV and  $106 \pm 0.2$  mV (mean  $\pm$  SEM). This oxidation which occurs with aging is also reflected in decreased levels of other antioxidants such as vitamin C or E.

these activities could lead to increased oxidative stress.

Although a causal relationship between oxidative stress and AMD has not been established yet, this association has been tested in several epidemiological studies (Christen, 1999; Snodderly, 1995). In our study, when the GSH redox state was compared between 40 AMD patients and 27 age-matched non-AMD individuals, there was a statistically insignificant trend toward lower total plasma GSH in the AMD subjects ( $p = 0.089$ ) (Fig. 3). High serum level of carotenoids is associated with reduced risk of neovascular AMD (Eye Disease Case-Control Study Group, 1993), while in the same study, no correlation was found between serum Vitamin C and E levels and AMD. Several factors limit the interpretation of these studies, including limited number of AMD patients and lack of adequate controls for other factors such as the antioxidant intake from food.

## 5. OXIDATIVE STRESS INDUCES APOPTOSIS IN RPE

Although accumulating evidence suggests a correlation between oxidative stress and AMD, a causal relationship based upon mechanistic studies of oxidative injury has not been established.

In vivo RPE studies are difficult to perform, not only because RPE accounts for only a single layer of the retina, but also due to the fact that a well-developed macula is limited to primate eyes.

To study the mechanism of oxidative stress induced injury, consequently, study of RPE in cell culture has been a major approach for mechanistic studies. Normal human RPE cells can be isolated from donor eyes and cultured in vitro. Tissues obtained within 24 h after death are usually easiest to culture but some success is obtained even after 48–72 h. RPE cells are considered to be postmitotic in vivo. However, when they are isolated from eyes and placed in culture with fetal bovine serum, they will start to divide for a limited number of passages (Flood *et al.*, 1980), probably due to both the stimulation by growth factors in the serum and the release from contact inhibition. These cells are useful for study of general cell senescence in vitro (Matsunaga *et al.*, 1999) as well as growth and injury. In vitro, cultured RPE cells still retain phagocytic function (Rakoczy *et al.*, 1996; Wassell *et al.*, 1998), can migrate (Hinton *et al.*, 1998) and can accumulate lipofuscin at high passage numbers (Holz *et al.*, 1999; Wassell *et al.*, 1998), all of which resemble their in vivo characteristics. However, their pigments are lost upon continued cell division unless they are constantly fed with rod outer segments. This loss of pigments renders them more resistant to light exposure (Wihlmark *et al.*, 1997).

We established an in vitro toxicity model using t-butylhydroperoxide (t-BHP) to generate oxidative injury to cultured RPE cells (Davidson *et al.*, 1994; Sternberg *et al.*, 1993). As a chemical oxidant, t-BHP is a relatively stable hydroperoxide and permeates cell membranes readily (Moore *et al.*, 1983). Once it is within the cell, it induces oxidative stress both by 2-electron oxidation and by metal ion and metalloprotein-catalysed free radical processes. It is detoxified by reduction to t-butanol by GSH peroxidase and this reaction is often associated with depletion of GSH (Comporti, 1987).

With several different RPE cell lines established from different donor eyes, t-BHP caused a dose-dependent cell death (Sternberg *et al.*, 1993; Cai *et al.*, 1999). At concentrations of 900  $\mu$ M,

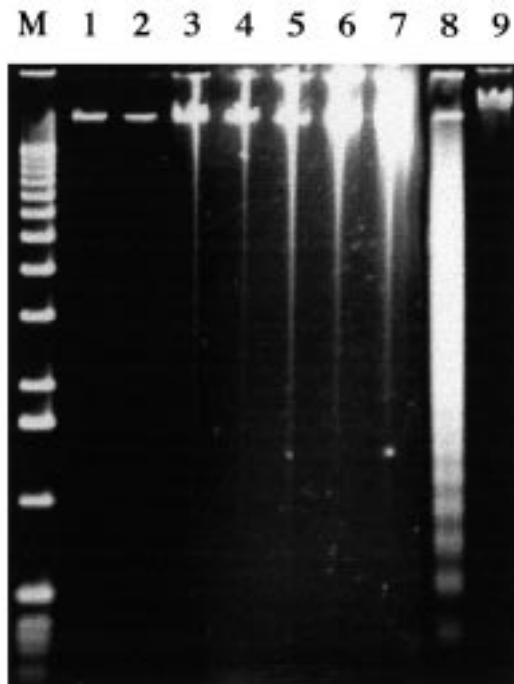


Fig. 4. Detection of DNA fragmentation in RPE cells treated with t-BHP. In vitro cultured human RPE cells were treated with 0, 50, 100, 200, 300, 500 and 900  $\mu\text{M}$  t-BHP for 10 h (Lanes 1–7). Genomic DNA was isolated and run on 1% agarose gels. Lanes 8 and 9 are HL 60 cells treated with 1  $\mu\text{M}$  staurosporine and control HL 60 cells, respectively. Although some DNA degradation can be seen in RPE cells treated with high concentration of t-BHP, the pattern is clearly different from that of apoptotic HL 60 cells.

most of the cells died within 2 h with cell swelling and with their membranes becoming permeable to the vital dye trypan blue, indicating acute necrosis. Similar results were obtained with RPE cells kept in suspension, except the sensitivity was higher (Sternberg *et al.*, 1993).

At lower concentrations of 300 and 500  $\mu\text{M}$ , t-BHP induced cell death with a much slower time course and with characteristics of apoptosis. These included nuclear chromatin condensation, cell shrinkage and caspase activation, all of which occurred before the loss of the plasma membrane integrity (Cai *et al.*, 1999). Unlike cells from the neurosensory retina, RPE cells do not show typical DNA fragmentation (Fig. 4), a process in which the genomic DNA is degraded

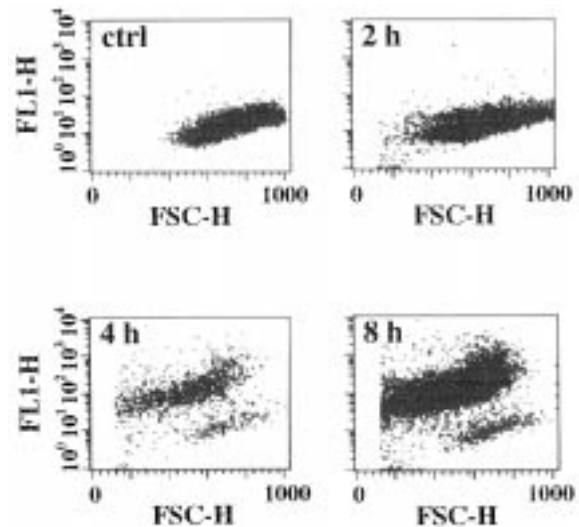


Fig. 5. Detection of oxidant-induced apoptosis in RPE cells with TUNEL labeling. Control and treated cells were assayed by flow cytometry after TUNEL labeling. Forward scattered height (FSC-H) is an indicator of cell size and a high fluorescence in the FL-1 region (FL 1-H) indicates TUNEL positive labeling. Apoptotic cells had higher FL-1 fluorescence and lower FSC height, which showed a distinct population after 4 h of t-BHP treatment. Results suggest that TUNEL labeling followed by flow cytometry analysis can be used to detect apoptotic RPE cells.

into fragments with size intervals of 180–200 base pair (Wyllie, 1980). However, DNA fragmentation into large fragments can be detected by TUNEL staining. Using flow cytometry, a distinct population with decreased cell size and increased TUNEL staining was detected as early as 4 h after 300  $\mu\text{M}$  t-BHP treatment (Fig. 5).

In vitro cultured RPE cells can be triggered to undergo apoptosis by a variety of other treatments, such as with experimental ischemia, protein kinase inhibitor, peroxynitrite, anti-FAS antibody and tumor necrosis factor (Behar-Cohen *et al.*, 1996; Jorgensen *et al.*, 1998; Kimura *et al.*, 1997; Osborne *et al.*, 1997). Therefore, like other cell types, the intrinsic apoptotic machinery is expressed within RPE cells and different stimuli can trigger it by different signaling pathways. However, the available evidence indicates that oxidative stress-induced apoptosis involves a signaling mechanism generated from mitochondria.

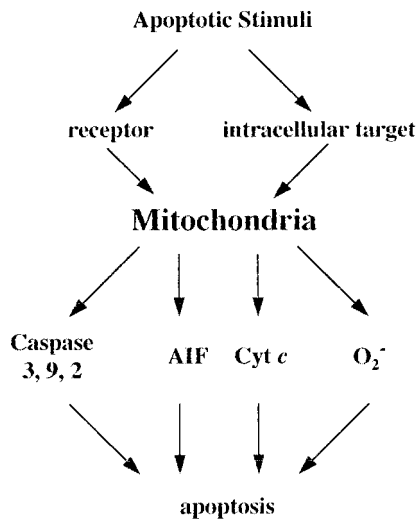


Fig. 6. Mitochondria play a central role in apoptosis. Different upstream apoptotic stimuli can be transduced to mitochondria and trigger the release of mitochondrial intermembrane proteins that function in signaling apoptosis. The mechanisms leading to their release are still under investigation. One possibility is through the mitochondrial permeability transition.

## 6. MITOCHONDRIA ARE THE TARGET OF OXIDATIVE INJURY

In t-BHP-treated RPE cells, an early decrease of mitochondrial membrane potential was observed before caspase activation and DNA fragmentation (Cai *et al.*, 1999). This sequential change suggests the involvement of mitochondria as the primary target of oxidative injury, an observation consistent with recent data showing that mitochondria have a central role in regulating apoptosis (Fig. 6) (Cai *et al.*, 1998; Yang *et al.*, 1997). The release of mitochondrial cytochrome *c* initiates the apoptosome formation in the cytosol and the subsequent caspase activation (Li *et al.*, 1997; Zou *et al.*, 1999). Together with cytochrome *c*, several other apoptogenic proteins are also localized to the mitochondrial intermembrane space and are released as a consequence of the breakdown of the mitochondrial outer membrane during early stage of apoptosis (Mancini *et al.*, 1998; Susin *et al.*, 1999a,b).

One mechanism by which mitochondria can release cytochrome *c* is to activate the mitochondrial permeability transition (MPT) pore. MPT is

a well studied phenomenon (Zoratti and Szabo, 1995) wherein the normally impermeable mitochondrial inner membrane abruptly becomes permeable to molecules of less than 2 kDa. Under normal physiological conditions, the MPT occurs as a consequence of the reversible opening of megachannels at the gap regions on the inner and outer membrane of mitochondria, which allows free distribution of ions across the inner membrane. Because of the very high inside-negative electrochemical gradient, opening of the permeability transition pore causes rapid inward ion movement, loss of mitochondrial membrane potential and extensive mitochondrial swelling. The whole process is usually reversible but the endogenous regulation and function remain unclear.

MPT has been proposed to play a critical role in regulating cytosolic  $\text{Ca}^{2+}$  concentration and to provide a mechanism for uptake and elimination of compounds for which no specific transport system exists (Ichas and Mazat, 1998). The molecular components of the MPT pore are being elucidated (Green and Reed, 1998). The core of the complex consists of porin on the outer membrane side and adenine nucleotide transporter (ANT) on the inner side, with peripheral benzodiazepine receptor and cyclophilin D associated with each one of them, respectively. One of the pro-apoptotic Bcl-2 family protein, Bax, has also recently been reported to interact with ANT directly (Marzo *et al.*, 1998).

A decrease of mitochondrial membrane potential, which could be due to prolonged, irreversible opening of the MPT pore, is found in many apoptotic systems. These include apoptosis induced by  $\text{TNF}\alpha$  and Fas, chemotherapeutic agents, oxidants, mitochondrial inhibitors, viral infections, growth factor withdrawal and a variety of toxins. Therefore, Kroemer and co-workers postulated that MPT is a common apoptotic 'effector' (Susin *et al.*, 1998a,b). As a consequence of the MPT, mitochondria release intermembrane proteins such as cytochrome *c* (Kantrow and Piantadosi, 1997).

Although the exact redox-sensitive site remains to be determined, the sensitivity of the MPT pore to calcium is modulated by the disulfide state of its vicinal -SH groups (Bernardi *et al.*, 1994).

Treatment of mitochondria with bifunctional sulfhydryl cross-linking agents, arsenite or phenylarsine oxide, potentiated the opening of the MPT pore in response to calcium (Chernyak and Bernardi, 1996; Costantini *et al.*, 1998; Lenartowicz *et al.*, 1996). It has been proposed that the key component on the inner membrane, adenine nucleotide translocator, has critical thiol groups, which upon oxidation, can facilitate the opening of the MPT pore (Majima *et al.*, 1995; Zoratti and Szabo, 1995). MPT may therefore represent a mechanism by which mitochondria sense the initial oxidative stress, amplify it and then transduce it to further downstream signals that lead to cell death, either by necrosis or apoptosis. The triggering of the MPT provides a possible explanation for how oxidative injury induces apoptosis in RPE (Cai *et al.*, 1999).

### 7. OXIDATIVE STRESS-INDUCED MITOCHONDRIAL DAMAGE ACCUMULATES WITH AGE

The sensitivity of mitochondria to oxidative injury could be modulated by the extent of previously accumulated mitochondrial damage. Mitochondrial macromolecules, including DNA, proteins and lipids, all can be the targets of oxidative damage. However, mtDNA is particularly sensitive to oxidative injury for several reasons. MtDNA is localized close to the source of ROS production, it is not covered by histones, it is a circular intron-less circular DNA with high transcription rate and the DNA repair system within the mitochondria appears to be less effective than that in the nucleus. MtDNA damage is particularly detrimental to non-dividing cells such as those in brain, heart skeletal muscles and RPE cells. In dividing cells, mitochondria replicate throughout the cell cycle, and abnormal ones can be lost by failure to replicate. However, in post-mitotic cells, abnormal mitochondria can accumulate due to failure to lose defective ones.

With *in vitro* cultured cells, a sublethal dose of hydrogen peroxide induced mtDNA damage that was much more extensive and was repaired more slowly than that for nuclear DNA (Yakes and van Houten, 1997). *In vivo*, both point mutations

and deletions have been detected in mtDNA. 8-oxo-2'-deoxyguanosine is an oxidative base modification product that has been used as a biomarker of oxidative DNA damage (Richter, 1995). HPLC measurements using samples from human diaphragm and brain showed the amount of 8-oxo-2'-deoxyguanosine significantly increased in mtDNA with aging and could reach 0.5 ~ 1% of total deoxyguanosine residues (Richter, 1995). Because other nucleotide modifications should also exist in addition to 8-oxo-2'-deoxyguanosine, the total amount of modified bases could be extensive and account for a significant proportion of total mtDNA.

Studies with long-extension PCR and high fidelity DNA polymerases to amplify nearly the whole mtDNA show that deletions of variable lengths are abundant in aging individuals, although such studies have not been carried out on RPE. In human skeletal muscle, both the number and variety of mtDNA deletions were found to increase significantly between young and old individuals (Melov *et al.*, 1995). Similar mtDNA deletions have also been detected in heart and brain of aged mice (Melov *et al.*, 1997). More importantly, none of the detected deletions was a predominant form, and the patterns of deletions varied from individual to individual. More recently, Ozawa and colleagues detected 358 different types of mtDNA in a 97 year-old subject with 'total-detection system' using 180 pairs of different PCR primers. They calculated that the remaining wild-type mtDNA without deletions only accounted for 11% of total mtDNA (Hayakawa *et al.*, 1996).

The susceptibility of mitochondria to oxidative injury, together with the age-related decrease of cellular anti-oxidant systems, provides the rationale for a mitochondrial based model of aging and age-related diseases (Harman, 1956; Shigenaga *et al.*, 1994; Wallace, 1999). As mentioned earlier, although certain genes have been identified to control aging in lower organisms, the aging process in higher organisms is much more complicated and probably involves multiple factors. Accumulation of damaged mitochondrial macromolecules due to oxidative process appears to be one of the important factors contributing to

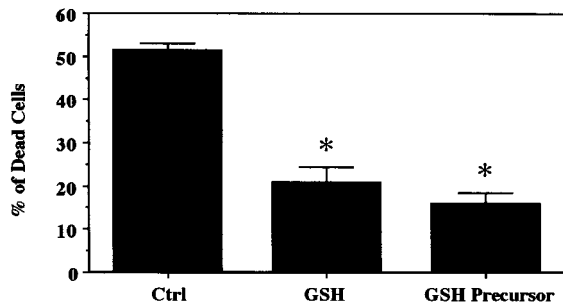


Fig. 7. Protection of RPE from oxidative injury by GSH. RPE cells in suspension were preincubated with either 1 mM GSH or 1.0 mM of amino acid precursors of GSH (glycine, glutamate and cysteine) for 10 min, treated with 150  $\mu$ M t-BHP for 2 h. (\* $p < 0.01$ ). Concentration dependence studies showed that micromolar concentrations of GSH were sufficient for protection while higher amino acid precursor concentrations were needed. These studies establish the principle that supplements can be used to enhance protection against oxidative injury in RPE.

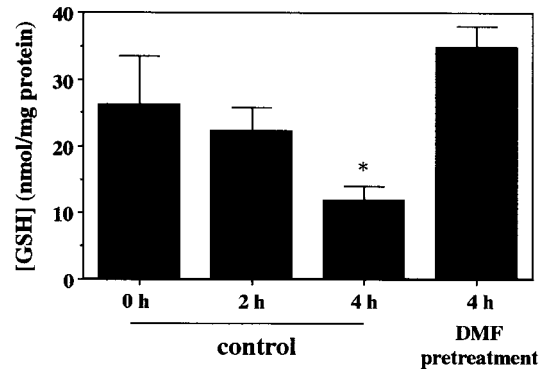


Fig. 8. Dietary phase II enzyme inducers which increase GSH synthesis rate protect RPE cells from t-BHP induced oxidative injury. Cells were treated with 100  $\mu$ M DMF for 24 h, followed by 300  $\mu$ M t-BHP treatment. The measured intracellular GSH indicates at 4 h time point, an early oxidation was induced by t-BHP and the protection of DMF was due to increased GSH synthesis. (\* $p < 0.05$ ).

aging and likely to be involved in the mechanism of AMD.

## 8. PREVENTION OF RPE LOSS BY ANTI-OXIDANTS

If oxidative stress is involved in the etiology of AMD, then the aging process of RPE and the development of AMD could be prevented or delayed by strengthening the antioxidant capacity of RPE. Although the results remain controversial, several epidemiological studies found correlations between increased intake of foods high in anti-oxidants and decreased risk of AMD. Higher dietary consumption of carotenoids, especially lutein and zeaxanthin from dark green, leafy vegetables, was found to be associated with decreased risk of AMD (Seddon *et al.*, 1994). Protection has also been associated with zinc, vitamin C and vitamin E, but these effects remain controversial (Mares-Perlman *et al.*, 1996; Richer, 1996). An on-going multicenter randomized clinical trial, Age-related Eye Disease Study (AREDS), is evaluating the role of antioxidant supplement in the progression of cataracts and AMD.

In addition to direct intake of antioxidants, induction of glutathione synthesis provides an

alternative way to protect the ocular antioxidant state. With in vitro cultured RPE cells, we have shown that at 0.5–1.0 mM concentration, a mixture of amino acid precursors for GSH synthesis, glutamate, glycine and cysteine, can significantly raise the intracellular GSH concentration (Davidson *et al.*, 1994). When added into the culture medium, the induction of synthesis occurred within 30 min. Preincubation of RPE cells with these three amino acids at concentrations higher than 0.1 mM protected the cells from subsequent t-BHP induced toxicity (Fig. 7). All three components were required for the protection. Cysteine alone was not protective and without cysteine, glutamate and glycine were not protective (Sternberg *et al.*, 1993).

GSH synthesis can also be induced by a number of naturally occurring compounds that induce phase II detoxification enzymes, including the rate limiting enzyme for GSH synthesis, glutamate cysteine ligase (Prochaska and Fernandes, 1993). We therefore studied the protective effect of the phase II inducer dimethylfumarate (DMF) on peroxide-induced damage on cultured human RPE cells (Nelson *et al.*, 1999).

DMF is rich in certain fruits, such as apples. It has relatively low toxicity even at large doses and is used as food additive (Spencer *et al.*, 1990). When cultured human RPE cells were treated

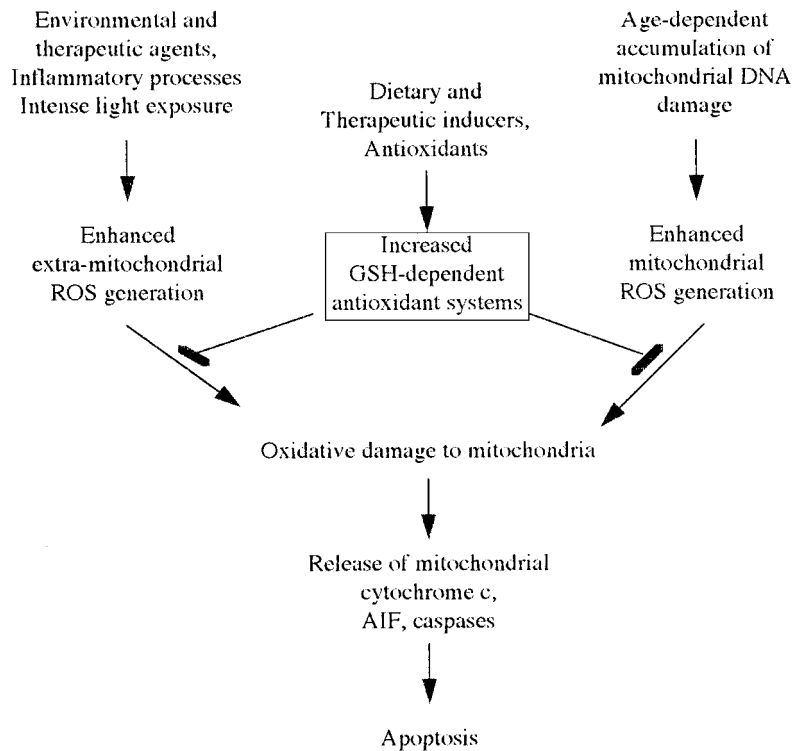


Fig. 9. Schematic model showing oxidative injury and protection of RPE. Different external and internal factors can damage mitochondria, induced ROS generation and lead to cell death. Dietary and therapeutic agents can exert their protect effect by enhancing the GSH antioxidant capacity of RPE.

with 200  $\mu\text{M}$  DMF for 24 h, the measured GSH concentration increased 2.5 fold. The increase of GSH was accompanied by a moderate increase in glutathione S-transferase activity; together, these increases provided a protection against subsequent peroxide-induced toxicity (Nelson *et al.*, 1999). Without DMF treatment, nearly all the cells died with 300–500  $\mu\text{M}$  t-BHP treatment in 24 h. Preincubation of the cells with DMF for 24 h protected them from t-BHP induced GSH oxidation (Fig. 8) and rendered them much more resistant to the t-BHP induced oxidative damage.

Although the *in vitro* data are promising, DMF also induces an early depletion of GSH due to the formation of a GSH-DMF conjugate (Nelson *et al.*, 1999). During acute exposure, the RPE cells showed increased sensitivity to t-BHP toxicity. It remains to be determined whether long-term treatment with DMF can overcome this initial drop of GSH and be useful therapeuti-

cally. Of particular importance, the *in vivo* effects of DMF need to be tested before it can be considered as a potential therapeutic agent. However, even if DMF can not be used clinically, other inducers of GSH synthesis have already been tested in pre-clinical trials and some of the phase II inducers are orders of magnitude more potent (Kensler *et al.*, 1993). It will be of interest to test whether some of them, such as oltipraz or sulforaphane (Benson, 1993), can increase GSH in RPE and protect the RPE cells from oxidative stress. Such an effect would indicate that phase II enzyme inducers may be useful to prevent or delay the onset of AMD, in addition to their chemopreventive effects on carcinogenesis.

## 9. FUTURE DIRECTIONS

Identification of genes whose function or ex-

pression is altered in AMD remains critical for understanding and preventing AMD. Newly developed high-output techniques, such as gene expression array assays, will facilitate this process (Berstein *et al.*, 1999). Functional analysis of the proteins encoded by those candidate genes, especially determining how they interact with the apoptotic pathway in RPE cells, will be essential in establishing their role in AMD.

However, the challenge remains great. AMD is a chronic disease. The available model systems, using either light exposure or chemical treatment, all involve an acute toxicity. The extrapolation of these data to explain the *in vivo* pathological conditions must be done with caution. New methods are needed to bridge this gap. Recently it has been found that RPE cells could be maintained as a confluent monolayer in culture for prolonged periods (Burke and Skumatz, 1998). Compared to dividing RPE cells, which are often used for model studies of oxidative injury, cells cultured under non-dividing condition are more relevant to their *in vivo* growth condition and could be a better system for chronic toxicity in the RPE.

The new information obtained from the DMF studies in RPE cells indicate that chemoprevention may not be limited to prevention of carcinogenesis, but also important in age-related disease. Further studies will be needed to determine whether phase II enzyme inducers, such as DMF and oltipraz, can elevate the GSH in retina and whether they can prevent oxidative damage *in vivo*. If so, these agents could provide an effective strategy to prevent or treat AMD.

In summary, individuals with different genetic backgrounds all face the burden of high oxidative stress in their RPE. Once the protection from the antioxidant system has been overwhelmed, apoptosis can be triggered by a mechanism involving the mitochondrial signaling (Fig. 9). Accumulation of sublethal chronic oxidative injury potentiates the oxidative injury on affected RPE cells. Antioxidant supplements, or other approaches to strengthen the antioxidant capacity of RPE, may delay the onset of age-related retinopathy.

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