# "OXIDATIVE PROTECTOR" ENZYMES IN THE MACULAR RETINAL PIGMENT EPITHELIUM OF AGING EYES AND EYES WITH AGE-RELATED MACULAR DEGENERATION*

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*Here I am, an old man in a dry month,*
*Being read to by a boy, waiting for rain.*
—TS Eliot, "Gerontion," 1919

## INTRODUCTION

Of all of the infirmities of age, blindness is perhaps the one most feared. Modern advances in ophthalmic therapy have been able to prevent, or to reduce the incidence of, severe visual loss in such diseases of aging as cataract and glaucoma, and both proliferative diabetic retinopathy and diabetic macular edema, which are major causes of visual loss in both younger and older individuals. Of the four ocular diseases reported by the Model Reporting Area Study of 1973 (which though nearly 25 years old remains probably the most accurate evaluation of data on blindness and visual loss throughout the United States) to be the major causes of visual loss in the US population over the age of 65,[1] age-related macular degeneration (ARMD) is the only one for which there are no effective means of prevention or treatment. Additionally, understanding of the pathogenesis of this disorder is limited, which poses a considerable hindrance to the rational development of measures to prevent or to treat it.

## HISTORY

Recognition of the lesions that we now consider to comprise the syndrome of ARMD is nearly a century and a half old, dating to Donders,[2] who in 1855 first described macular druse in subjects in the sixth decade of life and beyond. Multiple macular druse occurring in a familial pattern in younger individuals have also been described by many authors in the older

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literature.[3-6] These usually are inherited in an autosomal dominant pattern.[7] Although dominantly inherited druse in younger individuals most often appear clinically to be of the "soft" variety, while those in older subjects with ARMD may be of all of the different morphologic types (see below for classification of druse types), the histologic and electron microscopic appearances of "soft" druse in older and in younger individuals are indistinguishable.[8-10] The marked difference in age at onset and the usually clearly defined dominant inheritance pattern of druse in younger individuals by comparison with "soft" druse in ARMD suggests that the 2 disorders have a different etiology with a similar anatomic end point, but it is still not clear whether dominant druse in younger individuals represents simply one form of ARMD with an earlier onset and a much more evident inheritance pattern, or is an entirely different disease.

ARMD may progress in 2 ways: (1) toward "geographic atrophy" of the retinal pigment epithelium (RPE), choriocapillaris, and outer layers of the neurosensory retina or (2) toward the development of neovascularization arising from the choriocapillaris with serous detachment of the RPE and neurosensory retina, subretinal hemorrhage, and ultimate "disciform" scar formation. That these are different manifestations of the same, or very similar, disease processes seems apparent from observations of these different lesions in fellow eyes of the same patient (and, less frequently, in the same eye of a given patient). In 1885, Haab[11] originated the term "senile macular degeneration" to describe cases resembling the syndrome as we currently know it, including the various atrophic changes in the macula in addition to "disciform" lesions, although in recent years the name has been modified to "age-related macular degeneration" because the term "senile" usually connotes a deterioration of the mental status of an older individual.

Although it is a less common cause of visual loss in ARMD, choroidal neovascularization (the "wet" or "exudative" form of the disease) leading to disciform degeneration produces a more rapid, more dramatic, and more severe decrease in vision than the atrophic, or "dry," form and, probably for that reason, has received much more attention. Pagenstecher and Genth[12] were the initial observers to describe what was subsequently called the "disciform process,"[13] referring to the clinical appearance of an often round macular scar that was later recognized to comprise neovascularization arising from the choriocapillaris.[14] A more extensive description of this lesion was provided by Junius and Kuhnt in 1926,[15] and their names were applied eponymously to disciform degeneration of the macula. Ryan and associates[16] in 1980 provided an exhaustive review of the literature on the disciform process.

Atrophic lesions of the RPE, choriocapillaris, and neurosensory retina on an hereditary basis were described by Klien[17,18] and by Sorsby,[19] while

Gass,[20] in a thesis presented to this Society, first applied the term "geographic atrophy" to such enlarging lesions in ARMD because they had the appearance of regions on a map.

**DEFINITION**

Although there are a number of findings that most retinal clinicians at the present time would recognize as comprising the syndrome of ARMD, a precise definition does not exist.[8,21,22] Most clinicians would agree that macular druse, which usually appear as small, yellowish-white lesions at the level of the RPE that may be elevated and confluent and may also contain crystalline deposits, are an important feature of the disorder. Druse have been classified as hard, soft,[8,21] and also as mixed, basal laminar, or calcified.[21] A problem is that, in at least one study using standardized macular photographs and a grading scale with evaluation by readers masked to the identity of the subjects, 95.5% of the population greater than 43 years of age in the town of Beaver Dam, Wisconsin, had at least one macular druse,[23] as did over 80% of watermen from the eastern shore of Maryland over 30 years of age.[24] To formulate a definition of ARMD including druse as an essential finding, various investigators have required that either a certain number of druse be present[24] or the druse be principally "soft,"[23] or that druse must be present and also the visual acuity must be reduced to a certain level.[25] Other complexities, as noted above, include the fact that multiple, often "soft" and "confluent" macular druse have been observed in individuals much younger than the sixth decade, usually in families with an autosomal dominant pattern of inheritance.[4-7,9,10,20] Can these individuals be considered to have "age-related" macular degeneration? Finally, an increased incidence of disciform macular degeneration has recently been reported in elderly Japanese (cited by Bird[26]) and in elderly Greenland Inuit (Eskimos) (also cited by A.C. Bird, Symposium on Macular Diseases, University of Leipzig, Germany, November 14 and 15, 1996). As noted by Bird, affected individuals in both populations have disciform maculopathy but are never observed clinically to have druse. Yet those cases that have been examined histopathologically after death demonstrate basal laminar deposits between the RPE and Bruch's membrane in the macular region, considered to be a typical finding of ARMD.[8,27]

Other, later lesions of ARMD include macular hyperpigmentation, that is, hyperplasia of the RPE, in association with druse; "geographic" atrophy of the RPE and choriocapillaris; choroidal neovascularization; subretinal hemorrhage resulting from such neovascularization; and the formation of "disciform" scars. With the possible exception of large detachments of the RPE, none of these other lesions is unique to ARMD, and it is the constellation of findings in an older individual that usually leads to the diagnosis.

The modern study of ARMD began with the work of Gass,[20,28-30] who combined astute clinical observations on large numbers of patients with histopathologic descriptions and studies using the new (in the 1960s) technique of fluorescein angiography to provide a coherent picture linking the various stages of the disease with interpretations of the developing lesions at the cellular level. Gass demonstrated that druse, retinal pigment epithelial detachments, choroidal neovascularization, and hemorrhages with resultant disciform scarring, and also "geographic" atrophy of the RPE and choriocapillaris were all part of the spectrum of a single disease.

**PATHOGENESIS**

What was missing from this elegant work, and is still missing today, is an understanding of the pathogenesis of ARMD at the molecular level. This is more than just an intellectual challenge, since in the absence at the present time of effective therapy, save for the relatively few cases of neovascular ARMD that can benefit from laser photocoagulation,[31] precise knowledge of those cells in the eye that are first affected, and by what molecular processes the disorder begins, seems the most logical way to develop rational methods of prevention and of treatment.

Hypotheses of the tissue in which the first pathological processes of ARMD occur have varied widely and have included, as Gass[29] has pointed out, the inner retinal layers, the outer retinal layers, Bruch's membrane, the choriocapillaris, and the major choroidal vessels. More recently, several investigators have emphasized the role of aging changes in the RPE.[32-34] Most of these hypotheses have long since been discarded. A role for the photoreceptor cells in the pathogenesis of ARMD has recently been resurrected by the report of 13 different mutations in one allele of the ABCR gene in 26 (16%) of 167 unrelated ARMD patients.[35] ABCR (ATP-Binding Cassette Rim Protein), whose gene is found on chromosome 1, is a large-molecular-weight glycoprotein that serves as an ATP transporter. It is localized specifically to the rims of retinal rod outer segment discs,[36] and its homozygous mutation has been found to be responsible for Stargardt's disease (fundus flavimaculatus), a form of juvenile macular degeneration.[37] This fascinating finding is of interest for several reasons: First, because it suggests that a primary gene defect involving exclusively rod photoreceptors is responsible for a juvenile macular degeneration when it is present in homozygous form, and for at least some cases of a macular degeneration of somewhat different phenotype in much older individuals when it is present in heterozygous form. Second, the presence of the heterozygous ABCR mutation in only 16% of the ARMD subjects studied strongly suggests that the clinical entities that are together designated ARMD represent common morphologic end points of several etiologic pathways. This does not, however, obviate the hypothesis to be pro-

posed below and which is the basis for this thesis, since a multiplicity of initial biochemical lesions may act through a final common pathway (in this case, oxidative tissue damage) to produce a final common anatomic end point, namely, the lesions we recognize as ARMD.

Many investigators currently consider that ARMD originates in lesions of Bruch's membrane or the RPE.[8,26,29,32,33] Bruch's membrane is, however, a multi-layered extracellular structure derived from the RPE and the choriocapillaris. It has no metabolic activity, and its functions are largely passive, serving as a filter (or, conversely, as a barrier) for molecules passing between the RPE and choriocapillaris. The arguments for a primary pathogenetic role for Bruch's membrane in ARMD are based on alterations of these filtration properties with the deposition of druse and/or of basal laminar deposits beneath the macula of the aging eye[26,38,39] or, perhaps, the deposition of lipids within Bruch's membrane.[40] Like other basement membranes, the basement membranes of the RPE and of the choriocapillaris that comprise part of the pentalaminar structure of Bruch's membrane, and the other extracellular components of this structure, can also serve to modulate the differentiated functions of the adjacent RPE and choriocapillary endothelial cells. However, since all of the components of Bruch's membrane are synthesized either by the choriocapillary endothelium or by the RPE, abnormalities of the composition of Bruch's membrane must derive from pathological processes originating in the RPE or in the choriocapillaris, leading logically to the conclusion that the pathogenesis of ARMD must begin either in the choriocapillaris or in the RPE and not in Bruch's membrane per se. The hypothesis on which the present study is based, however, is that the primary lesion responsible for ARMD (or at least, responsible for the final common pathway to ARMD) occurs in the RPE. This single layer of cells is highly metabolically active and carries out multiple functions, of which probably only a small number are currently known. These include phagocytosis of the tips of photoreceptor outer segments to maintain these specialized structures in an appropriate state of renewal[41]; storage of retinol (vitamin A alcohol) in esterified form as a reservoir for visual pigment renewal in the photoreceptors[42]; oxidation of retinol to retinal (also called retinaldehyde, the chromophore of all known visual pigments), and stereoisomerization of retinal to its appropriate form for visual pigment synthesis, which is also stored within RPE cells bound to a specific protein, cytoplasmic retinal-binding protein (CRALBP)[42,43]; transport of oxygen and other nutrients from the choriocapillaris to the avascular outer layers of the retina, and transport of waste products in the reverse direction; all the while maintaining an appropriate barrier function between the bloodstream and the neural retina[44]; participating in the formation of the interphotoreceptor matrix at the apex of the cell and of a portion of Bruch's membrane at the

cellular base[45]; and performing a metabolic pumping function to help maintain the attachment of the neural retina to the apex of the RPE and of the choriocapillaris/Bruch's membrane to its base.[46] It is reasonable to suppose that this multiplicity of functions in a single layer of cells with minimal regenerative properties is highly taxing, and that these functions deteriorate with age, particularly in the maculas of humans and higher primates, which are the most visually (and, one would presume, metabolically) active regions of the retinas of these species. Whatever the functional consequences of alterations in Bruch's membrane, the hypothesis on which the present study is based holds that these alterations, as well as the other abnormalities that comprise the clinical syndrome of ARMD, take place subsequent to the occurrence of primary biochemical lesions in the macular RPE.

What could those lesions be?

## THE FREE RADICAL HYPOTHESIS OF AGING

One prominent hypothesis of aging holds that oxidative damage to cells, initiated by free radicals, is a major cause of the aging process.[47-49] According to this hypothesis, enzymes that destroy free radicals, such as superoxides, and also reactive species such as peroxides, protect cells against age-related damage. Over time, irreversible tissue damage caused by such oxidative processes accumulates to produce the phenotypic changes that we recognize as "aging." In the presence of specific risk factors, which may be genetic, environmental, or a combination of both, such oxidative stresses may lead to pathologic changes that we recognize as disease. Such risk factors may include a genetic deficiency or malfunction in one or more of the "oxidative protector" enzymes; dietary deficiency of, or inability to use appropriately, antioxidant substances provided exogenously; or exposure to noxious environmental influences (?smoking). Although observations consistent with this hypothesis have been made in several lower species,[49-51] the totality of evidence at present is not conclusive.[49] However, there is evidence of a role for toxic oxidative processes in several systemic diseases of humans, and these processes have also been investigated for their possible involvement in disease in a number of tissues in the eye.

*Studying the Role of Oxidative Damage in Tissues and Diseases.* The study of potentially damaging oxidative processes in tissues has been pursued using several methodologies. One way is simply to measure the levels of presumed toxic oxidative agents such as peroxides, superoxides, and other free radicals, as well as other molecules (such as lipid peroxidation products, measured as thiobarbituric acid reactive substances or "TBARS") in the tissues under investigation. Because these molecules are relatively labile and short-lived, they are not satisfactory for the type of

study that will be reported here, in which tissues obtained postmortem are shipped often over long distances with at least many hours elapsed between time of death and arrival of the tissue in the laboratory. A second method, which is best suited for clinical trials in human subjects or studies on colonies of intact laboratory animals, is the use of various antioxidants as chemopreventive agents for the disease processes under investigation. Finally, there is the method to be used in the present study, which involves measuring levels of several different enzymes that are thought to protect tissues against oxidative damage from certain toxic molecules. This method assumes that, if those antioxidant enzymes that are constitutively expressed are present in the relevant tissues in higher levels, the tissues will be afforded greater protection against oxidative damage, while individuals who for genetic or other reasons have lower levels of one or more of these enzymes will be less well protected. However, one of the enzymes to be studied here, heme oxygenase-1 (HO-1), is reported to be induced by the presence of oxidative "stress." As will be discussed, there is some question as to whether this enzyme actually does protect tissues from oxidative damage, but its presence at high levels is considered evidence that such stress is present.

The enzymes to be studied here include copper, zinc-containing superoxide dismutase (Cu,Zn-SOD), catalase, glutathione peroxidase, HO-1, and HO-2 (an enzyme that is structurally and functionally closely related but immunologically distinct from HO-1, and which is constitutively expressed). All of these enzymes are primarily found in the cell cytoplasm. This study will not consider manganese-containing superoxide dismutase (Mn-SOD), which is located in the mitochondria. A diagram of the function of these enzymes in cells is presented in Fig 1. The superoxide dismutases convert ("dismute") oxygen free radicals ("superoxides") to peroxides, which are themselves further broken down either by nonenzymatic processes that produce, in turn, hydroxyl free radicals, or by enzymatic processes involving glutathione peroxidase, peroxidase, or catalase. The heme oxygenases convert heme, a powerful oxidant produced by the breakdown of hemoglobin or of cytochromes within the mitochondria to biliverdin, which is then further reduced to bilirubin, an excellent antioxidant, by the action of biliverdin reductase (Fig 1). The induction of HO-1 by oxidative stress has been reported in many tissues and species,[52-54] but this response may not be entirely beneficial.[53] For example, the action of the heme oxygenases to reduce heme to bilirubin also produces free $Fe^{2+}$ and carbon monoxide (Fig 1), both of which can be damaging to cells.

*The Role of Oxidative Damage in Human Systemic Diseases.* High doses of oral antioxidants, including vitamin E and probucol, have been shown to be effective in preventing coronary artery occlusions and restenosis of coronary artery bypass grafts in several clinical trials.[55,56] Free

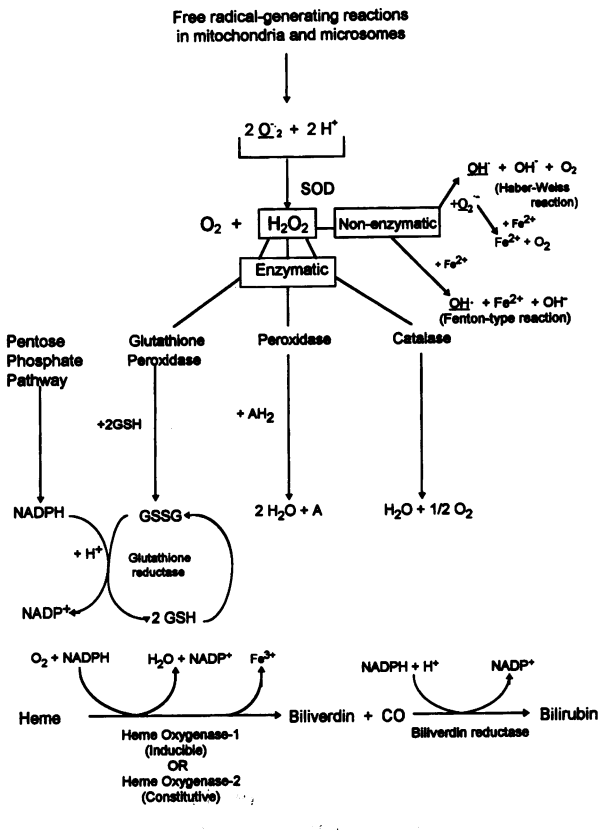


FIGURE 1

Cellular reactions involved in breakdown of free radicals and peroxides, and which protect the cell against oxidative damage. Free radicals (indicated in diagram by underlining) are generated principally within mitochondria and microsomes, but also within cytoplasm. They are initially broken down ("dismuted") by the Cu,Zn-containing form of SOD in cytoplasm or by the Mn-containing form within mitochondria. (Because this diagram depicts the general scheme of these reactions, Cu,Zn-SOD and Mn-SOD are not distinguished from one another here.) Hydrogen peroxide, formed by this enzymatic process, may be broken down either by nonenzymatic processes or, more rapidly, by enzymatic reactions including peroxidase (not studied here), glutathione peroxidase, or catalase, all within the cytoplasm. Additionally heme, from the breakdown of hemoglobin in erythrocytes or cytochromes in mitochondria, is converted to biliverdin and then to bilirubin by actions of heme oxygenases and biliverdin reductase.[52]

*Adapted, with permission, from Berman ER, Biochemistry of the Eye. New York: Plenum Press; 1991:39.*

radicals produced by oxidative reactions have been claimed to induce abnormalities of vascular endothelial cells in experimental diabetes in animals, with the implication that similar abnormalities are present in human diabetes mellitus and may be in part responsible for the late complications of that disease.[57,58] There have been numerous studies of the relationship of serum and tissue levels of various antioxidants, and of antioxidant supplementation, to the pathogenesis, prevention, and treatment of several types of human cancer. Several very large controlled clinical trials in this area are still ongoing,[59,60] but no results have yet been reported. Finally, at least one important neurodegenerative disease associated with aging in humans has been associated with abnormalities of enzymes involved in the detoxification of of oxygen free radicals: the familial form of amyotrophic lateral sclerosis, which in a number of kindreds has been linked to genetic defects in Cu, Zn-SOD.[61]

*The Role of Oxidative Damage in the Eye, Exclusive of the RPE.* Toxic products of oxidation including peroxides and free radicals, such as superoxides, have been considered important as causal agents of various pathological processes in other tissues in the eye in addition to the RPE. The possibility that ARMD begins with a biochemical abnormality in the neural retina, perhaps in its outer layers, has been discussed here in particular with regard to the possible role of mutations in the ABCR gene in some cases of this disease. The photoreceptor outer segments, consisting of stacks of lipid-rich membranes, are subject to peroxidative reactions, and the accumulation of potentially damaging lipid peroxides with aging has been considered in these structures.[62] Biochemical and immunocytochemical studies have described in various layers of the retina many of the enzymes involved in protection of tissues from oxidative damage, including catalase,[63-65] glutathione peroxidase,[64,66] Cu,Zn and Mn-SOD,[64,65,67] and the heme oxygenases.[68,69] There is some discrepancy in various reports regarding precise localization of these enzymes to specific retinal layers, especially the photoreceptor outer segments, which in many enzymologic studies in the past have been found to be relatively devoid of most enzyme activities. This is also true of more recent reports, save for Cu,Zn-SOD, which in at least one of the present studies has been described immunocytochemically in photoreceptor outer segments,[65] while in another study the outer segments were devoid of this enzyme,[67] and for glutathione peroxidase, which one group of investigators has described in photoreceptor outer segments of rats and rabbits.[66] One study[64] measured several different enzyme activities in trephined retinal buttons from the maculas and superotemporal peripheries of human cadaver eyes from inviduals ranging in age from 7 to 85 years. There was wide interindividual variation and, with the exception of SOD activity (only total activity was measured with no distinction between the Cu,Zn- and Mn-dependent forms), no consis-

tent variation with donor age was found. For SOD, activity in the peripheral retinal buttons declined significantly with age ($P = 0.04$), but this was not true for activity in the macula. Although glutathione peroxidase activity in both macular and peripheral retina showed no age-related trend, the difference between macular and peripheral activity decreased significantly ($P = 0.025$) with increasing age. Given the wide individual variations in activity and the fact that neither activity in the peripheral retina nor activity in the macula showed a consistent trend with age, the apparent age-dependent trend in the difference between the two activities may not be biologically meaningful.

In addition to ARMD and, possibly, diabetic retinopathy, other disorders in which the antioxidant defense system of the retina, perhaps aided by exogenous antioxidant supplementation, has been considered to be possibly beneficial include retinal ischemia resulting from vascular occlusion,[70,71] proliferative vitreoretinopathy,[72] and, most controversially, retinopathy of prematurity.[73-76] Initial reports that administration of vitamin E, an antioxidant, to very-low-birthweight premature infants suppressed the development of the severe proliferative forms of this disease[73,74] were followed by much larger, controlled clinical trials[75,76] showing that this treatment was not significantly beneficial and also produced an increased incidence of severe and life threatening complications, including necrotizing enterocolitis and sepsis.

There have been a few descriptions of antioxidant enzymes in the cornea, including heme oxygenase in human corneal epithelium,[77] Cu, Zn-SOD in corneal epithelium and endothelium of humans and several other species,[78] and catalase in corneal endothelium of rabbits.[79] The role(s) of these activities in protecting the cornea against the oxidative stresses of age, disease, inflammation, and chronic light exposure have not been studied extensively, certainly not to the degree that they have been investigated in other tissues of the eye. There have been no studies of the relationship of oxidative protector enzyme activities in the cornea either to aging or to specific disease states either in humans or in animals.

Studies of antioxidant enzymes in the iris and ciliary body have also been few. A biochemical study in rabbits indicated that these tissues had the highest specific activities of glutathione peroxidase of any ocular tissue examined.[80] Immunocytochemical studies from one laboratory[63,67,81] reported catalase, Cu, Zn-SOD, and glutathione peroxidase activity in rat iris pigment epithelium and ciliary body, but a very recently published report[82] using immunogold electron microscopic labeling of Cu, Zn-SOD, glutathione peroxidase, and glutathione S-transferase in human eyes showed Cu, Zn-SOD and acidic glutathione S-transferase antigen in all cells of the iris, while glutathione peroxidase and basic glutathione S-transferase was present in erythrocytes only. There have been no quantitative studies of

antioxidant enzymes in the iris or ciliary body, and no attempts to relate enzyme activity or antigen presence to aging or to disease.

Cataract and open-angle glaucoma are disease processes that might be influenced by the inability of the relevant tissue — the lens and the trabecular meshwork — to defend itself against oxidative insult. There have been extensive studies of various "oxidative protector" enzymes in the lens. Assays of SOD, glutathione peroxidase, and catalase in the nuclei and cortexes of a series of 76 surgically excised human cataractous lenses showed no consistent changes in catalase activity with progression of the cataract, but a very substantial decrease in SOD and glutathione peroxidase with progression of nuclear cataracts, to which the authors ascribed possible causal relevance.[83] By contrast, assay of enzymes including glutathione peroxidase, glutathione reductase, and 6-phosphogluconate dehydrogenase in a series of 174 lens epithelium-capsule specimens from human patients who had undergone cataract surgery showed no relationship to the type or severity (evaluated by a standardized photographic grading system) of cataract.[84] Age-related changes in enzyme activity in the lens have also been investigated. Glutathione peroxidase activity increased and glutathione reductase activity decreased with age in 3 species of simians (orangutan, olive baboon, and pigtail monkey), but glutathione peroxidase activity decreased or remained relatively constant in 2 species of prosimian (galago and mouse lemur).[85] Yet another study in clear human lenses obtained postmortem indicated a decrease in SOD activity with age, but an accumulation of inactive enzyme protein detected by immunoassay.[86] Nearly all investigators attribute some role in human cataractogenesis to oxidative injury, based on animal or in vitro studies with cultured lenses in which enzymes have been inhibited, on results demonstrating that lenses from transgenic mice with increased expression of an oxidative protector enzyme (glutathione peroxidase) showed delayed cataractogenesis while lenses from mice in whom there was a targeted disruption ("knockout") of the gene for this enzyme had more rapid cataract development,[87] and, finally, on the well-known fact that the aqueous humor has an unusually high concentration of ascorbate (vitamin C), a powerful water-soluble antioxidant.[79] However, translation of these findings into clinical efficacy in cataract prevention has not yet occurred. Perhaps the use of antioxidant vitamins as cataract preventive agents in the ongoing Age-Related Eye Disease Study, sponsored by the National Eye Institute, will prove beneficial.

The pathogenesis of chronic open-angle glaucoma, which, like cataract and ARMD, is an age-related eye disease, is unclear. But age-related changes in oxidative protector enzymes of the trabecular meshwork that might make this tissue susceptible to damage leading to decreased filtration merit consideration. Activities of SOD and catalase per milligram wet

weight of tissue in calf eyes were comparable to those of retina and higher than specific activities in lens,[88] while in human eyes, SOD activity showed a highly significant ($P = 0.00022$, $r^2 = 0.64$) decline with age, but catalase did not ($P = 0.24$, $r^2 = 0.16$).[89]

*The Role of Oxidative Damage in the Pathogenesis of ARMD.* Several investigators have suggested that the free radical hypothesis of aging applies to ARMD,[90-92] in particular because the central area of the neurosensory retina and the underlying RPE may be susceptible to the cumulative toxic effects of continued photic damage. A number of reports from one group of investigators have described studies in the RPE of the enzymes catalase, Cu,Zn-SOD, and mitochondrial manganese-containing superoxide dismutase (Mn-SOD),[93-95] all of which are involved in the breakdown of toxic free radicals and peroxides. Most notably, Liles and associates[94] assayed excised RPE from human donor eyes for catalase and SOD. They found diminished catalase activity with age in both macular and peripheral retinas of normal subjects. Individuals with either macular or peripheral RPE abnormalities had decreased catalase activity relative to normal subjects, but showed no greater age-related decrease than did normal subjects. Overall SOD activity (in this report, as in most of the others previously cited, the investigators did not distinguish between Cu,Zn-SOD and Mn-SOD) was said to show no difference between normals and diseased tissue, and no statistically significant change with age. In this study, 6 of 14 "diseased" eyes had ARMD, based on the finding of druse when the posterior ocular segment was examined by the dissecting microscope, while the other 8 eyes had "peripheral disease," not further defined. Some researchers have investigated "oxidative protector" enzymes in red blood cells rather than in retinal tissue itself for a possible relationship to ARMD. Results of these studies have been conflicting,[96,97] and this approach does not appear to the present author to be fruitful, since the molecular defects responsible for ARMD would seem most likely to reside in the affected tissue itself.

Two other approaches to exploring the pathogenesis of ARMD, and the possible validation of the free radical hypothesis, have been employed in studies of animal models and in epidemiologic surveys of human populations. Certain kindreds of cynomolgous monkeys have been found to develop a disorder of aging resembling ARMD, including druse-like deposits beneath the RPE and histopathologic lesions resembling the human disease. In such animals, Nicolas and colleagues[98] reported significantly increased levels of albumin and decreased levels of glyceraldehyde 3-phosphate dehydrogenase within the retinas of affected monkeys by comparison with controls. Though their reasoning is not entirely clear, they concluded that "degradative as well as antioxidant enzymes might be involved in the mechanisms leading to macular degeneration . . . the

results also . . . appear to indicate that oxidative stress is significant in the etiology of early onset macular degeneration." Perhaps more relevant, though occurring in a species lacking a macula or a substantial number of cone photoreceptors, is the observation that the oxidative protector enzyme HO-1 is markedly up-regulated in the retinas of rats exposed to intense visible light (the rat is an important model of retinal light toxicity), and this induction is prevented by injecting the rats with the antioxidant dimethylthiourea.[53] Because, as we have seen, HO-1 is induced by oxidative stress, it appears to be an important marker for determining the presence of such stress in tissues and the response of these affected tissues to it. The role of these enzymes in the retina[53] and RPE[99,100] has only recently begun to be explored. There have as yet been no studies of the heme oxygenases in retinal or RPE tissue from humans as a function of aging or disease.

Epidemiologic studies relevant to the role of toxicity of products of oxidation in the RPE include the following: (1) ARMD, and in particular its most severe forms, is much more common in white than in black populations.[101-103] (2) ARMD has been reported by some investigators to be more prominent in individuals with light-colored irides than in those with dark irides,[104,105] and another group[106] has claimed that the disease is more prominent in individuals whose irides grew lighter in color with age. The Eye Disease Case-Control Study, however, failed to confirm these findings, at least with regard to neovascular ARMD.[107] If ARMD is closely related to skin and ocular pigmentation, it could be considered to be etiologically similar to skin malignancies and other diseases that are thought to be produced though excessive sunlight exposure. (3) Sunlight exposure was proposed as a possible cause of ARMD by Young.[92] While some studies[108,109] have yielded positive results at least with regard to visible light (ultraviolet light, which appears not to be causally related,[110] is also likely irrelevant since it is largely absorbed by the ocular media, specifically including the lens, in phakic and most present-day pseudophakic eyes before it reaches the retina), others[104,111] have not. (4) The carotenoids lutein and zeaxanthin, consumed in the diet, particularly in certain dark-green, leafy vegetables, appear to be protective against neovascular ARMD.[112] Supplemental estrogen therapy (which may have an antioxidant effect) is also protective in postmenopausal women.[107,113] Cigarette smoking, however, is detrimental.[114,115]

The hypothesis upon which the present study is based, derived from the information already summarized here, is that the initial molecular lesion(s) resulting in ARMD occur(s) within the RPE and involve(s) one or more of the "oxidative protector" enzymes. Because these lesion(s) most likely predominate in the RPE beneath the macular retina rather than throughout the retina including its periphery, the method of choice

for conducting this study was quantitative electron microscopic immunocytochemistry, which permits investigation of relative concentrations of particular proteins in individual cells, or even cellular organelles, or in very limited regions of tissue. This method was of particular use in studying proteins within RPE cells of choroidal neovascular membranes that had been removed surgically from ARMD patients because these membranes are very tiny, and isolating and studying biochemically the small population of RPE cells within them by other methods would have been extremely difficult. Biochemical studies, though much more crude in their ability to dissect out enzyme activities in small regions of RPE, were employed in this investigation in an attempt to give some confirmation of the quantitative immunocytochemical data. Biochemical assays also provided a way to test the validity of using donor eyes that, even in the best of circumstances, had to be shipped long distances and therefore could not be processed in the laboratory until many hours after death.

## METHODS AND MATERIALS

### HUMAN TISSUE SPECIMENS

The experiments described herein for electron microscopic immunocytochemical studies used 21 whole human eyes obtained postmortem from donors aged 42 to 94 years at the time of death, including 6 males and 15 females. When a pair of eyes was received from the same donor, quantitative immunocytochemistry was carried out on one eye, chosen at random. In 2 cases (indicated in Table I) in which both eyes of one donor were sent to the laboratory, the fellow eye was used for cell culture. However, there were 15 additional donor eyes from individuals ranging from 40 to 88 years of age and including 5 males and 10 females, listed separately in Table I, in which cell culture studies only were carried out. These eyes were obtained from eye banks around the United States through the National Disease Research Interchange (NDRI), Philadelphia, Pa. The protocol that this laboratory submitted to the NDRI requested that eyes be sent as soon as possible after the death of the donor, though in some cases there was a delay as long as 48 hours. Eyes were sent on ice by express mail, and fixation for electron microscopic immunocytochemistry or procedures for the isolation of RPE cells for culture and biochemical studies did not begin until after the arrival of the eyes in the laboratory. Table I lists the donors, their ages, race, sex, and causes of death as reported by the local eye banks to the NDRI, and the time, in hours, between death and the initiation of preparation of the eyes in the laboratory either for immunocytochemistry or for cell culture and biochemical studies.

Nineteen of the whole human eyes (from 6 males and 13 females) used

in these immunocytochemical studies were judged to be normal based on examination of the posterior globes under the dissecting microscope following receipt in the laboratory and removal of the cornea, lens, iris, and vitreous. Two of the eyes (both from females) used for immunocytochemical studies had disciform lesions of ARMD as evaluated by this examination. Table I also indicates whether or not a disciform lesion was present in these eyes. Substantial numbers of macular druse were not observed among the specimens examined in this study. Of the 15 pairs of eyes used exclusively for cell culture, 9 were judged to have normal retinal and choroidal examinations and 6 had evidence of severe macular degeneration. The preponderance of female subjects, among both donors of normal eyes and whole eyes with severe ARMD, and also among the surgical cases that will be described, is noteworthy and cannot readily be explained. Despite efforts to obtain eyes from individuals of different racial groups, all but 1 of the 36 individuals who donated eyes that were obtained from NDRI for use in these studies were white. Details are given in Table I.

Additionally, 19 specimens of choroidal neovascular membranes (CNVMs; from 7 male and 12 female patients) were obtained immediately following pars plana vitrectomy procedures by a single surgeon at the author's medical center for excision of such CNVMs resulting from ARMD. Seventeen of these patients were white, while 2 male patients were black. Ages ranged from 55 to 92 years. Patients had signed informed consent both for the surgery and for the use of the excised tissue (which in these procedures is not submitted for routine histopathology) in research studies. Both the surgical procedure and the research studies to be described had been approved by the Human Investigations Committee at the author's medical center. Surgical techniques have been described in several previous publications.[116-118] As will be described, these specimens were placed immediately in fixative and taken to the laboratory to be prepared for electron microscopic immunocytochemical studies.

No attempt was made to match normal control eyes and eyes, or surgical specimens, with CNVMs by age. Nevertheless, a reasonably good match was obtained. Fig 2 demonstrates histograms of the numbers of eyes or surgical specimens of CNVMs by decade of age among specimens obtained from normals and from individuals with disciform ARMD, both in the group used for immunocytochemical studies and in the group used for cell culture/biochemical experiments. Comparisons by several types of contingency table analysis showed no statistically significant differences between control and CNVM subjects by decade of age in either group. (For the specimens used in immunocytochemical studies, $X^2 = 2.98$, $P = 0.70$; log likelihood $G^2 = 3.77$, $P = 0.58$; Monte Carlo randomization using 1,000 "shuffles" yielded $P = 0.80$. For the specimens used in biochemical

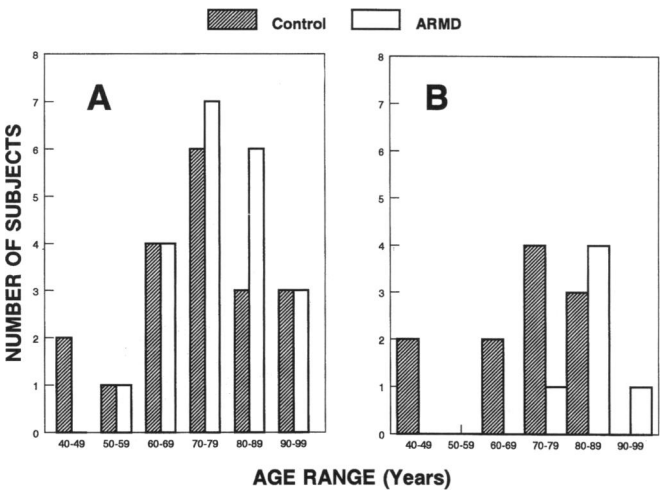


FIGURE 2

Histograms showing distribution of ages of subjects in this study, by decade. A, Subjects whose whole globes or surgically excised CNVMs were used for quantitative electron microscopic immunocytochemistry. B, Subjects whose whole globes were used for RPE cell culture and biochemical assays. As noted in text, age differences by decade between control and ARMD eyes were not statistically significant, either in immunocytochemistry or in biochemistry groups, when compared by several types of contingency table analysis.

experiments, $X^2 = 5.99$, $P = 0.20$; log likelihood $G^2 = 7.51$, $P = 0.11$; Monte Carlo randomization using 1,000 "shuffles," $P = 0.22$. All P values are two-tailed.)

#### ANIMAL TISSUE SPECIMENS

Calf eyes were obtained from a local slaughterhouse for use in some of the cell culture and biochemical studies to be described. These eyes had been obtained within 2 hours of the death of the animal, usually in batches of 50 eyes, and brought on ice immediately to the laboratory. The purpose of the experiments with bovine eyes was to determine how enzyme activities in RPE cells varied with time after death, either by assaying bovine RPE cells for activity of the different enzymes studied here after removing the cells from fresh ocular specimens, by keeping the intact eyes in the refrigerator at 4° C for varying time periods before performing these assays, or by isolating RPE cells for culture either from fresh eyes or from eyes kept in the refrigerator for varying time periods.

**ELECTRON MICROSCOPIC IMMUNOCYTOCHEMISTRY**

*Antibodies*

The following antibodies and dilutions were used: A polyclonal antibody (IgG) to bovine erythrocyte Cu,Zn-SOD, prepared in rabbits, was obtained from Rockland Laboratories, Gilbertsville, PA. It was used in a dilution of 1:200. A polyclonal antibody to bovine CRALBP, also prepared in rabbits, was a gift from John C. Saari, PhD, Department of Ophthalmology, University of Washington, Seattle. The characterization of this antibody has been described.[43] It was used in a dilution of 1:200. A polyclonal rabbit anti-human erythrocyte catalase, and a polyclonal sheep anti-human glutathione peroxidase were purchased from Biodesign, Kennebunkport, ME. A polyclonal antibody to HO-1 was prepared in rabbits to the rat testicular antigen, and a polyclonal antibody to HO-2 was prepared in rabbits to the rat liver antigen. Both were purchased from StressGen Biotechnologies, Victoria, BC, Canada. Save for anti-CRALBP, which is not commercially available at present, the characterization of these antibodies has been described by their suppliers. Except for anti-CRALBP and anti-Cu,Zn-SOD, all other antibodies were used at dilutions of 1:100 using 20 mM Tris-buffered normal saline (TBS), pH 7.4 with 1% bovine serum albumin (BSA), as the diluent. All of the studies described here were carried out using the same lots of primary and secondary antibodies. It was not possible to perform immunostaining on all specimens in the same session both because of the numbers of specimens involved and because the specimens were acquired over many months. However, the antibodies were diluted and then aliquotted upon initial receipt in the laboratory, and aliquots were stored frozen at -20° C until use. All of the specimens studied in this investigation were not immunostained using the full panel of antibodies. This was done in part because of the small size of some of the surgical specimens and in part simply because of the magnitude of the project, given the numbers of antibodies and of surgical and eye-donor specimens. While the selection of specimens to be studied by a particular antibody was not randomized by any formal scheme, no selection process was involved that might have resulted in a systematic bias.

*Technique*

In the operating room, surgically excised tissues were immediately placed in a fixative solution consisting of 3.5% paraformaldehyde, 0.5% glutaraldehyde in 50 mM phosphate-buffered normal saline (PBS), pH 7.4, at 22°C. Fixation was carried out for 4 hours, after which the tissue was washed, dehydrated, and embedded in LR White resin (Polysciences, Warrington, PA) and cured for 12 hours at 22°C under an ultraviolet lamp ("Blak-Ray," UVP, Inc, San Gabriel, CA), then ultrathin sectioned. Upon arrival in the laboratory, whole eyes that were being prepared for electron

microscopic immunocytochemistry had an incision made in the cornea to allow intraocular penetration of the fixative, following which they were placed in the fixative described above for 4 hours at 22°C, then washed in 3 changes of PBS. The cornea, iris, lens, and vitreous were removed and the posterior retina was examined under the dissecting microscope to determine the presence of any abnormalities. The macular region was then chopped into pieces approximately 5 mm square, which were then dehydrated in graded alcohols, and embedded in LR White resin. For electron microscopic immunocytochemistry, ultrathin sections mounted on 200-mesh nickel grids were pre-incubated with blocking solution (TBS, containing 1% bovine serum albumin [BSA] and 5% normal goat serum [Sigma Chemical Co, St Louis, MO]) for 30 min. at 22°C. Sections were floated on droplets of primary antibody at the appropriate dilution for 2 hours at 22°C. The sections were then washed with TBS containing 0.1% BSA and incubated for 1 hour at 22°C with a 1:40 dilution of the secondary antibody in TBS, pH 8.2, and containing 0.1% BSA. The secondary antibody was goat antirabbit IgG conjugated to 10 nm colloidal gold particles, purchased from Amersham, Arlington Heights, IL, or, when the antigen was glutathione peroxidase, goat antisheep IgG conjugated to 10 nm colloidal gold (EY Laboratories, San Mateo, CA). After incubation with the secondary antibody, sections were rinsed with TBS and then with distilled water and air-dried. They were counterstained with uranyl acetate before being viewed with a transmission electron microscope.

In each series of immunochemical preparations, control sections were included in which either nonimmune rabbit or sheep IgG (obtained from Sigma) substituted for the primary antibody. Control sections were incubated with nonimmune IgG for 2 hours at 22°C, washed 3 times in TBS, and incubated with the secondary antibody. Sections were then washed, stained with uranyl acetate, and viewed by transmission electron microscopy.

*Morphometry*

This was similar to methods that have been described elsewhere.[119,120] Ultrathin sections were cut approximately perpendicular to the plane of the retina, or apically to basally for surgically excised CNVMs (as best could be judged in these tiny pieces of tissue, blocks prepared from which were marked on embedding). These were examined with a transmission electron microscope, photographed at a magnification of approximately 6,000X, and printed at a final magnification of about 20,000X. The magnification of the electron microscope was calibrated weekly. It was not critical that each photomicrograph be enlarged to precisely the same magnification, so long as the final magnification was known for accurate calculation of colloidal gold particle counts per unit area. Using a digitizing

tablet (HiPad, Houston Instruments, Houston, TX), a microcomputer, and a digitizing program (BioQuant, R& M Biometrics, Nashville, TN), the total RPE cell area on each micrograph was measured and from it were subtracted the areas of all RPE cell nuclei and lysosomes observed on the micrograph. Colloidal gold particles were then counted over the cytoplasm and lysosomes separately in each micrograph. This was done because initial observations suggested that there were large differences between labeling of cytoplasm and lysosomes for the different proteins that were being evaluated in this study. Nuclei (which did not appear on each section) were arbitrarily excluded from this analysis, and mitochondria and melanosomes were arbitrarily included with the cytoplasm. (Mn-SOD, which is known to be a mitochondrial enzyme,[93] was evaluated biochemically but not immunocytochemically in this study). One or more sections were examined from each of at least 2 blocks per specimen for each protein evaluated. It was difficult to examine a greater number of sections for the surgically excised CNVM specimens, since they were usually extremely small. Even had more tissue been available, it would have been necessary to "step" through the block to obtain multiple sections for analysis for each protein, because statistical evaluations for the same antigen from serial or near-serial sections are of doubtful validity, being equivalent to deriving statistics from multiple measurements of the same section. However, the RPE cell cytoplasmic area present on each photomicrograph was of the order of 50 $\mu m,^2$ which is substantially larger than the unit of measurement chosen for this study, gold particles/$\mu m,^2$ thereby strongly suggesting that the tissue area per individual section, and the number of sections counted per specimen, was quite adequate to yield valid statistical determinations. The mean number of gold particles/$\mu m^2$ could be calculated easily from the raw counts and area measurements using the statistical software program. In the surgically excised specimens, and in those whole eyes that contained them, RPE cells from submacular neovascular membranes were examined in this fashion. In normal eyes, the examination was carried out on RPE cells from the macular region, defined as extending 5 mm temporally from the temporal border of the optic nerve, and vertically between the superior temporal and inferior temporal major vascular arcades

Statistical analyses were carried out using the True Epistat software program, version 5.3 (Epistat Σ Services, Richardson, TX).

#### ENZYME HISTOCHEMISTRY

This was performed to identify lysosomes, which are positive for acid phosphatase activity. The method of Novikoff[121] was used. Ultrathin sections of tissue fixed as already described were incubated with Novikoff's medium, which contains 25 mg cytidine 5'-monophosphate (Sigma) as the

substrate and lead nitrate ($PbNO_3$) at a final concentration of 3.6 mM to produce the electron-dense reaction product in a total volume of 25 mL containing 0.05 M acetate buffer, pH 5.0. Incubation was carried out for 1 hour at 37°C, and the grids containing the sections were washed, counterstained with uranyl acetate, and viewed with a transmission electron microscope.

**CELL CULTURE**

Bovine and human RPE cells were cultured in identical fashion following removal of the neural retina from the posterior eye cup and incubation of the eye cup with a mixture of 0.01% collagenase (Gibco/BRL, Grand Island, NY) and 25 U/mL Dispase (Collaborative Research, Bedford, MA) for 45 to 60 minutes at 37°C. The enzyme solution was then removed and the RPE cell layer was detached from Bruch's membrane by gentle trituration using a sterile Pasteur pipette. Following washing 3 times with Hanks' balanced salt solution (HBSS; Gibco/BRL), the RPE cells were cultured in 35-mm-diameter plastic culture dishes (Falcon Plastics, Division of Becton Dickinson, Lincoln Park, NJ) on E-C-L Matrix (a basement membrane matrix prepared from the murine Engelbreth-Holm-Swarm tumor; Upstate Biotechnology, Lake Placid, NY) using Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 100 μg/mL gentamicin (all from Gibco/BRL). At confluence, the cells were passaged using 0.05% trypsin (Gibco/BRL) with 0.02% EDTA in HBSS without calcium or magnesium. Release of the cells from the culture dish usually took 4 to 10 minutes at 37°C. Because of the amount of cell protein required for enzyme assays, cells for second-passage cultures were plated on larger (60-mm-diameter) culture plates. When the cells became confluent, the contents of one such plate permitted two determinations of each of the enzyme activities described below. The author found, as reported by Campochiaro and Hackett,[122] that cultured RPE cells grow more readily as an epithelioid monolayer with a morphology like RPE cells in vivo when they are plated on a basement membrane matrix, although melanin pigment is lost with successive cell divisions. In addition to their morphology, RPE cells can be recognized because they stain with antibodies to CRALBP.[43] Homogeneity of the cultures was assessed by scanning multiple medium power fields through the tissue culture microscope at the centers and peripheries of the monolayers to detect cells that differed morphologically, or by randomly selecting cultures for immunostaining and assessing the proportion of cells that did not take up the specific stain. The culture methods described here produced almost completely homogeneous (>95%) RPE cell cultures. The very small proportion of contaminants seemed unlikely to invalidate the results of the experiments described here.

All enzyme assays were carried out on morphologically well-differentiated (epithelioid) second-passage cultures. The cells were gently scraped from the culture dish with a rubber "policeman," washed 3 times in PBS, pH 7.2, then sonicated 3 times at 50% power using a Branson Sonifier/Cell Disruptor Model 185 (Branson Ultrasonics, Danbury, Conn) for 5 seconds each and centrifuged at 3,500 x g and 4°C in a DuPont Sorvall MC 12V microfuge using an F-12/M18 rotor (Sorvall Instruments Division, DuPont, Inc., Wilmington, DE). Aliquots of the supernatant were used for all enzyme assays. Enzyme assays were normalized to the protein content of the supernatant, measured using the bicinchoninic acid method.[123] As described, this method did not produce errors due to the presence of cell membrane proteins or proteins from the extracellular matrix substrate, which are insoluble and hence were sedimented in the sonication and centrifugation procedure described.

**ENZYME ASSAYS**

Unless otherwise described, all biochemical reagents were obtained from Sigma. Inorganic salts were obtained from Fisher Scientific, Pittsburgh, PA.

*Superoxide Dismutase*

The method was that of Kuthan and associates.[124] The assay mixture contained 10 $\mu$M cytochrome C, 0.1 mM xanthine, variable amounts of RPE cell supernatant, 50 $\mu$M KCN (to inhibit cytochrome C oxidase possibly present in the tissue suspension), 0.1 mM EDTA, and 50 mM phosphate buffer, pH 7.2 in a total reaction volume of 1 mL. The reaction was initiated by addition of 0.5 U xanthine oxidase and followed at 25°C in a recording spectrophotometer at 550 nm to monitor the reduction of cytochrome C as long as the reaction rate remained linear. All reagents were purchased from Sigma. SOD activity is defined as the amount of protein in the tissue sample required to reduce the reaction rate by 50%. Since SOD reduces the amount of superoxide needed for cytochrome C reduction, the smaller the amount of protein required, the greater the activity. For this reason, in the plots of SOD activity to be presented below, activity is given as the reciprocal of the amount of protein to decrease the reduction of cytochrome C by 50%, so that larger numbers represent greater activity. Cu,Zn-SOD activity is distinguished from that of Mn-SOD by adding to the reaction mixture 2 mM KCN, which inhibits Cu,Zn-SOD, leaving only Mn-SOD. The difference between the total activity (in the presence of only 50 $\mu$M KCN) and the residual Mn-SOD activity represents the activity of Cu,Zn-SOD.

*Catalase*

The method used was that of Beers and Sizer.[125] The reaction mixture (1.5

mL total volume) contained 20 mM $H_2O_2$ in 17 mM phosphate buffer, pH 7.0. The reaction was initiated in a recording spectrophotometer by adding an aliquot of RPE cell supernatant and was followed at 240 nm and 25°C as long as the reaction rate remained linear. A unit of activity was defined as: $[(\Delta A_{240}$ per min) X 1,000]/(43.6 X mg protein per mL reaction mixture).

*Glutathione Peroxidase*

Enzyme activity was assayed as described by Stone and Dratz.[126] Glutathione peroxidase is present as selenium-dependent and non-selenium-dependent forms. Total glutathione peroxidase activity was determined using a reaction mixture which contained, in a total volume of 1.2 mL including RPE cell supernatant, 0.21 mM GSH (reduced glutathione), 0.4 U GSSG (oxidized glutathione) reductase, 0.1 mM NADPH, and 50 mM Tris-HCl, pH 7.2 with 0.1 mM EDTA. The reaction was initiated by adding 2 mM cumene hydroperoxide. Activity of the selenium-dependent enzyme was determined by substituting 0.1 mM $H_2O_2$ and 2.6 mM $NaN_3$ (to inhibit catalase) for the cumene hydroperoxide. After addition of tissue supernate, the reaction was followed at 25°C in a spectrophotometer at 340 nm, with activity expressed as nmol NADPH oxidized/mg protein/min. Non-selenium-dependent activity was obtained by subtracting the selenium-dependent activity from the total. For comparison, sonicates of the cytosol released from fresh rat liver obtained from animals killed during the course of other experiments in the laboratory were also assayed.

*Heme Oxygenases*

The method was that of Lincoln and associates,[127] which measures the production of bilirubin by the enzyme biliverdin reductase, which is present in substantial excess in many tissues, including the RPE. (This was demonstrated in other experiments in the author's laboratory, not reported here, in which gel electrophoresis was carried out on sonicated human RPE cell cultures. Blots of the gels onto nitrocellulose sheets were stained using an antibody to biliverdin reductase.) Heme oxygenase is the rate-limiting step in the conversion of heme to bilirubin,[52-54] the first reaction of which is the reduction of heme to biliverdin. The assay mixture, in a total volume of 0.25 mL, contained an aliquot of tissue supernatant, 25 $\mu$M hemin (Sigma), prepared as 10 mM hemin in dimethyl sulfoxide diluted to 250 $\mu$M hemin in 0.15 mM BSA, 40 mM Tris-HCL, pH 7.6. An aliquot of hemin solution comprising one-tenth volume of the total was added to nine-tenth volumes of the assay mixture to initiate the reaction. The reaction mixture also contained 1 mM NADPH and 15 µM BSA in 100 mM phosphate buffer, pH 7.4. After adding the hemin substrate the reaction was allowed to proceed for time intervals up to 60 minutes at 37°C, at

which times it was stopped by the addition of p-hydroxymercuribenzoate to a final concentration of 1 mM. The reaction tubes were centrifuged at 10,000 X g for 2 minutes in a microfuge, and the absorbance of the supernatants was read at 465 and 540 nm. Bilirubin formation is determined by its absorbance at 465 nm, whereas 540 nm is considered a baseline wavelength. Absorbance at 540 nm is subtracted from that at 465 nm to yield the net absorbance of bilirubin. Molar conversion was made using an extinction coefficient of 43.5 $mM^{-1}$ $cm^{-1}$. Measurements at the intermediate times up to 60 minutes showed that the reaction was linear both with respect to time and to protein content of RPE cell sonicate, at least for this period. Making this assumption, activity was expressed as the rate per minute using the value obtained at the conclusion of the incubation. To differentiate between HO-1, the inducible form, and HO-2, the constitutive form of the enzyme, some culture plates were incubated overnight before sonication and assay with 10 $\mu$M sodium arsenite in 1% fetal bovine serum and activity was compared in plates incubated with, and those without, arsenite, which induces HO-1.

## RESULTS

### IMMUNOCYTOCHEMISTRY

*Cu,Zn-SOD*

Fig 3 illustrates representative electron micrographs of specimens of normal macular RPE cells from a donor eye of an 85-year-old woman (Fig 3A), and RPE cells from a CNVM removed surgically from a 63-year-old woman with neovascular ARMD (Fig 3B). Both have been immunostained using anti-Cu,Zn-SOD as the primary antibody. Fig 3C shows a control section from the specimen also illustrated in Fig 3A, in which nonimmune rabbit IgG replaced the primary antibody. (To conserve space, control sections for the other antibodies are not shown.) Fig 3D shows a section from RPE cells from the eye of an 83-year-old woman with ARMD that has been stained with a lead ion technique for acid phosphatase. Similar to Novikoff's initial description,[121] the micrograph of Fig 3D demonstrates heavy labeling around the perimeter of the cellular structures that appear morphologically to be lysosomes. Cellular morphology is clearly demonstrated, although the technical quality of these electron micrographs, which were prepared for immunoelectron microscopy, is less than would have been the case for tissues fixed in more concentrated glutaraldehyde or osmium tetroxide solutions for conventional electron microscopic viewing. Evidence that the immunostaining is specific is, first, that it is almost entirely confined to cellular structures (in Figs 3, A and B, arrowheads designate representative colloidal gold particles in the cytoplasm and lysosomes as well as "background" labeling over spaces between cells) and, second, that there is no staining when nonimmune IgG is substi-

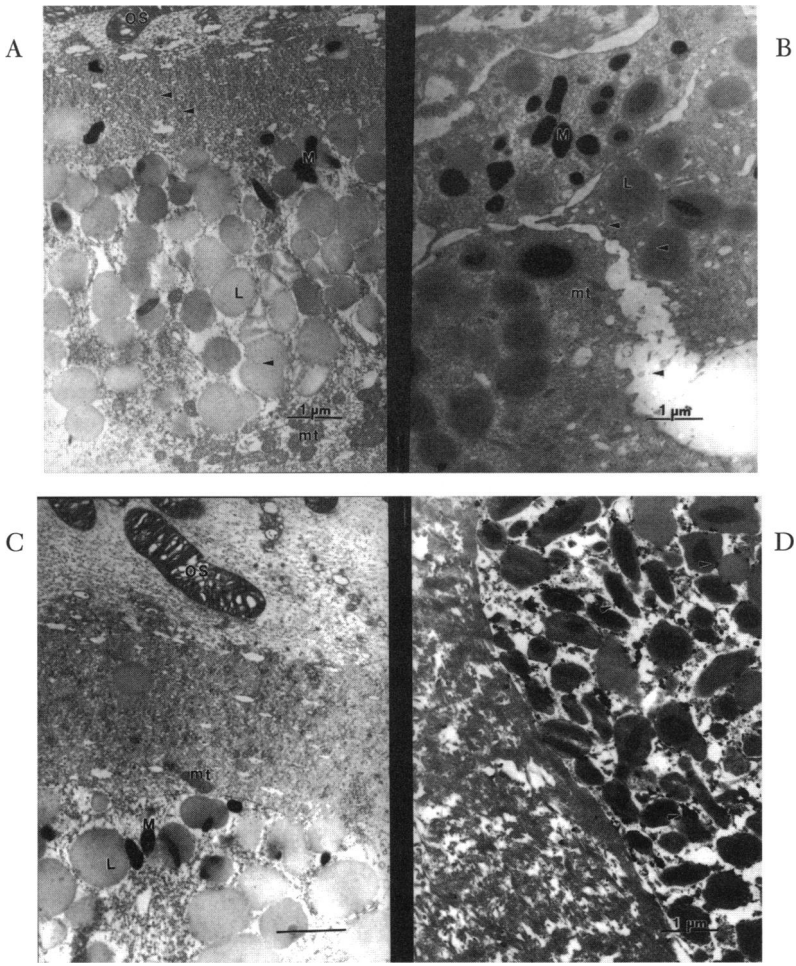


FIGURE 3

Immunostaining for Cu, Zn-SOD. A, Electron micrograph of RPE cells from macular region of donor eye from 85-year-old woman without evidence of ocular disease. B, Electron micrograph of RPE cells from CNVM that had been removed surgically from 63-year-old woman with neovascular ARMD. C, Control section from eye shown in Fig 3B, in which nonimmune rabbit IgG replaced primary antibody. D, Section showing portion of RPE cells from CRNVM from 83-year-old woman that has been histochemically stained with lead ion technique to demonstrate acid phosphatase. Although there is some background staining, most of labeling (arrowheads) is along peripheries of structures labeled L in Figs 3A-C, similar to results initially reported by Novikoff.[121] In these photomicrographs, M = melanosomes; L = lysosomes; mt = mitochondria. Arrowheads indicate representative colloidal gold particles in cell cytoplasm, in lysosomes, and in free spaces where there are no cellular processes (these latter demonstrate level of "background" staining). These labels are presented in this figure to denote certain cellular structures and organelles, but are not repeated in subsequent photomicrographs. Calibration bars = 1 $\mu$m in these micrographs and in all of the subsequent photomicrographs in this thesis.

tuted for the primary antibody. Fig 4 shows plots of the mean (±1 standard deviation [SD]) gold particle counts/$\mu m^2$ for the cytoplasm and lysosomes of these specimens. For clarity, this is broken into 2 plots: Fig 4A demonstrating gold particle counts for the normal donor specimens, and Fig 4B for the ARMD specimens (including both surgical specimens and donor eyes containing macular CNVMs). These figures use identical sets of axes and are aligned horizontally to permit comparison of the plots for cytoplasmic, and for lysosomal, labeling of normal and ARMD specimens. Although exponential functions fit the data somewhat better (that is, their r values are higher), the best linear fit is shown, together with the $r$ values for these plots, because these permit calculations of the significance of the differences of the plots from each other, and from a line of zero slope, which would indicate no influence of age on SOD labeling. Multivariable logistic regression using normal versus ARMD as the outcome variable and age, sex, cytoplasmic Cu,Zn-SOD labeling, lysosomal Cu,Zn-SOD labeling, and hours between death and preparation of the tissue in the laboratory as the independent variables showed that none of these was related significantly to the presence of a submacular CNVM from ARMD. (For these comparisons, hours between death and preparation for the donor eyes were derived from information provided to us by NDRI and the individual eye banks that supplied the donor eyes; for surgical specimens from the author's hospital, this interval was arbitrarily chosen as 1 hour, since it was never more than this.) Of the variables tested, only cytoplasmic labeling was of borderline significance ($P = 0.07$, but with an odds ratio of 0.92 and a 95% confidence interval of 0.83 to 1.01). The plots appear to indicate greater cytoplasmic than lysosomal labeling, since the best-fitting cytoplasmic plots lie above the best-fitting lysosomal plots in both figures, and at least some of the individual pairs of data points (shown by asterisks) show significantly greater cytoplasmic than lysosomal labeling. However, statistical comparison of the slopes and elevations of the cytoplasmic versus lysosomal plots in Figs 4, A and B, show no significant differences (P values for the difference in slopes are 0.83 and 0.73 for Figs 4A and 4B, respectively, and for the elevations the values are borderline at $P = .098$ and $P = 0.12$, respectively). The slopes of all of the plots are positive, however, showing increased Cu,Zn-SOD immunoreactivity with age in both cytoplasm and lysosomes of RPE cells of both normal and CNVM specimens. Only the $r$ value for the slope of the cytoplasmic labeling versus age plot in ARMD specimens shows a significantly positive value, however, with $r = 0.63$, $P = 0.011$.

*Catalase*

Catalase immunolabeling showed a pattern rather similar to that of Cu,Zn-SOD, although "background" labeling appeared to be considerably less (Fig 5). As with Cu,Zn-SOD, labeling of cellular cytoplasm both in RPE cells from normal eyes and from CNVMs was greater than that of lysoso-

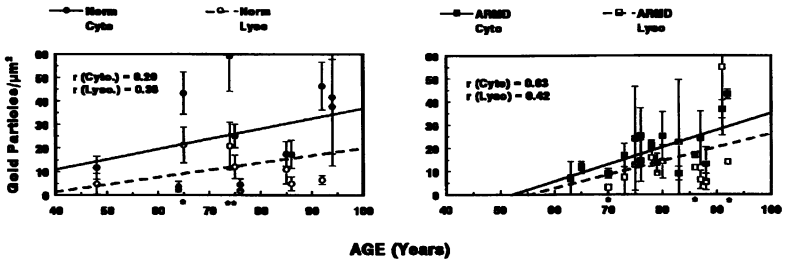


FIGURE 4

Mean (± 1 SD) gold particle counts/μm² for specimens immunolabeled with a polyclonal antibody against Cu,Zn-SOD, plotted as a function of the age of the subject. Left, RPE cells from normal donor eyes; Right, RPE cells from surgical specimens and donor eyes with CNVMs due to ARMD. Since both plots represent immunostaining against same primary antibody and plots use identical sets of axes, they may be directly compared. In both plots, filled symbols represent cytoplasmic gold particles while, open symbols represent lysosomal gold particles. The *r* values for best-fitting linear plots (which are illustrated in figures) are shown. Asterisks indicate pairs of values for cytoplasmic and lysosomal labeling of tissue from individual specimens that differ significantly with $P \leq 0.05$ using Student paired *t*-test.

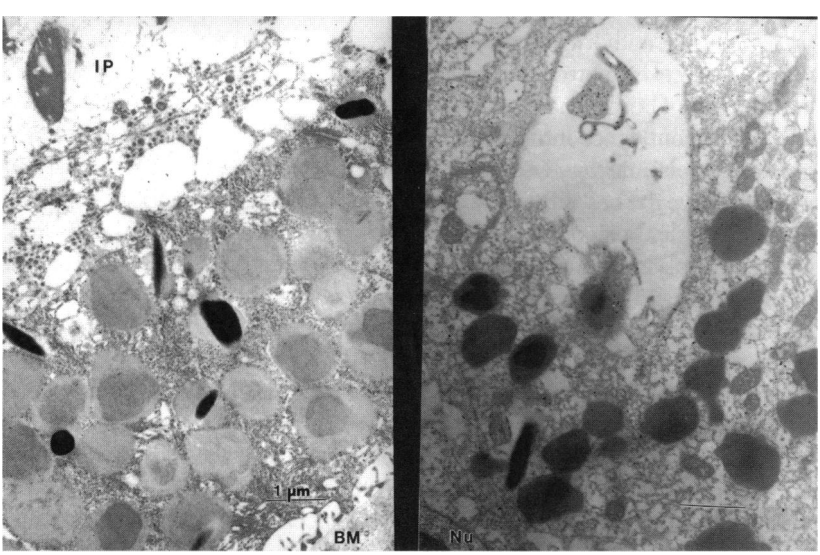


FIGURE 5

Immunostaining for catalase. A, Section through the macular RPE of a 79-year-old man with normal-appearing eye. There is moderate labeling of cytoplasm and lysosomes, but also some "background" labeling of vacuolar spaces and of interphotoreceptor matrix (IP) and of Bruch's membrane (BM). B, Section through a CNVM from an 86-year-old man with neovascular ARMD. There is virtually no labeling outside of cellular structures, although some labeling is seen in portion of cell nucleus (Nu) seen at lower left of figure. Calibration bars = 1 μm.

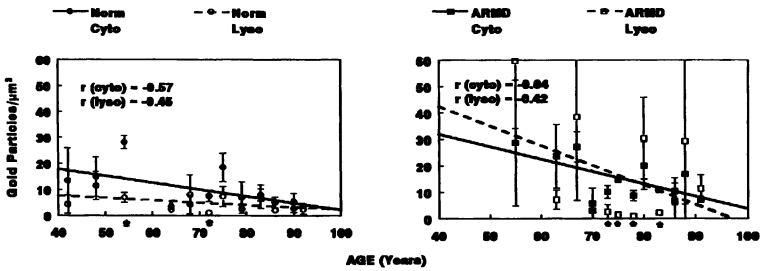


FIGURE 6

Mean (± 1 SD) gold particle counts for specimens immunolabeled with a polyclonal antibody against catalase, plotted as function of age of subject. Left, RPE cells from normal donor eyes. Right, RPE cells from surgical specimens and donor eyes with CNVMs due to ARMD. As in Fig 2, since both plots represent immunostaining against the same primary antibody and the plots use identical sets of axes, they may be directly compared. In both plots, filled symbols represent cytoplasmic gold particles while open symbols represent lysosomal gold particles. The *r* values for best-fitting linear plots (which are illustrated in figures) are shown. Asterisks indicate pairs of values for cytoplasmic and lysosomal labeling of tissue from a particular specimen that differ significantly with $P \leq 0.05$ using Student paired *t*-test.

mal labeling. This is demonstrated graphically in Fig 6. Many more pairs of points for cytoplasmic and lysosomal labeling in the same specimen differ significantly (asterisks) than in Fig 4. However, comparison of the curves for cytoplasmic and lysosomal labeling showed a significant difference only for the plots for normal eyes, with no significant difference in the slopes of the curves but a significant difference in their elevations, with $P = 0.027$. By contrast with the plots for Cu,Zn-SOD, the plots for catalase showed decreasing immunogenicity as a function of age. The *r* values for the best-fitting linear plots were significant for cytoplasmic labeling, with $r = -0.57$ and $P = 0.05$ for normal eyes and $r = -0.64$, $P = 0.025$ for CNVMs in eyes with ARMD. Multivariable logistic regression using normal and ARMD as the outcome variable and age, sex, cytoplasmic labeling, lysosomal labeling, and hours from death to preparation of the tissue in the laboratory as independent variables showed no significant correlation of any of these variables to outcome.

*Cytoplasmic Retinaldehyde-Binding Protein (CRALBP)*

RPE cells from both normal macular retinas and from CNVMs labeled very heavily for CRALBP, with very little background labeling (Fig 7). This, of course, does not mean that there is relatively more CRALBP antigen in these cells than there is Cu,Zn-SOD or catalase, since the intensity of the immunostain relates not only to the quantity of antigen

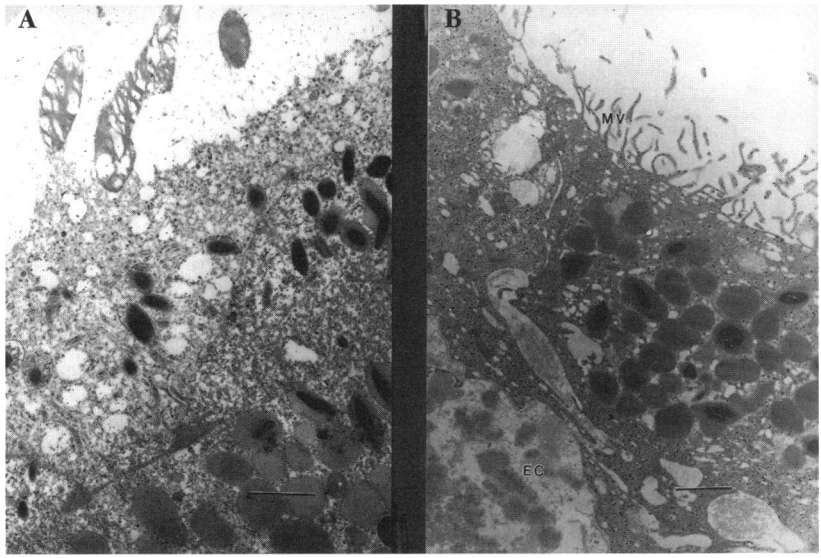


FIGURE 7

Immunostaining for CRALBP. A, Section through macular RPE of 64-year-old man with normal eye. There is very extensive labeling of cytoplasm and very little lysosomal labeling, with minimal "background" labeling. B, Section through CNVM from 55-year-old man with neovascular ARMD. Note prominent apical microvilli (MV) in these RPE cells, even though they have been detached from neural retina in this pathological specimen, and also thickened and irregular extracellular matrix (EC) at base of these thinned RPE cells. Strong evidence for specificity of this immunostain is heavy labeling of thinned microvilli with no visible background labeling of empty space in this section surrounding cellular apices. Note that, in this and other figures (eg, Figs 1 and 3), RPE cells even in normal sections are of varying thickness, since in photomicrographs taken at comparable (though not identical) magnifications, the entire thickness of the RPE cell is visible in some sections, while in others only a portion of cell is seen even though it was attempted to cut the sections as nearly as possible along a perpendicular plane between the apex and base of cell. (This cannot, of course, always be done precisely, especially in CNVMs, where tissue orientation in the block is always difficult.) Calibration bars = 1 $\mu$m.

present but also to the avidity of the particular antibody for that antigen and to the amount of colloidal gold linked to the secondary antibody. It must be emphasized that the comparisons made in this thesis deal only with the relative quantities of each antigen that are present in RPE cells of different individuals and in ARMD and normal eyes as well as with the relative amounts of labeling of each antigen in different cellular structures, and not with quantitative comparisons of labeling of one antigen with that of another. Examination of Fig 7, A and B, shows an apparent substantial difference between immunolabeling for CRALBP of cytoplasm and lysosomes in both normal and ARMD specimens, with extensive cytoplasmic labeling but very little labeling of lysosomes. This is

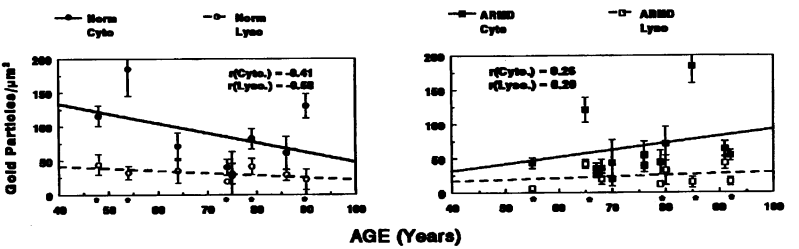


FIGURE 8

Mean (± 1 SD) gold particle counts for specimens immunolabeled with polyclonal antibody against CRALBP, plotted as function of age of subject. Left, RPE cells from normal donor eyes. Right, RPE cells from surgical specimens and donor eyes with CNVMs due to ARMD. As in Figs 2 and 4, since both plots represent immunostaining against same primary antibody and plots use identical sets of axes, they may be directly compared. In both plots, filled symbols represent cytoplasmic gold particles and open symbols represent lysosomal gold particles. The *r* values for best-fitting linear plots (which are illustrated in figures) are shown. Asterisks indicate pairs of values for cytoplasmic and lysosomal labeling of tissue from a particular specimen that differ significantly with $P \leq 0.05$ using Student paired *t*-test.

confirmed in the plots of Fig 8, which show the average cytoplasmic and lysosomal counts of gold particles/$\mu m^2$ as a function of age for both normal (Fig 8A) and ARMD (Fig 8B) specimens. Paired values for cytoplasmic and lysosomal counts for the same specimen for which grain counts/$\mu m^2$ differ significantly are indicated by asterisks. The slopes of the best-fitting straight lines for cytoplasmic and lysosomal counts are shown, and do not differ significantly from one another, but the elevations are highly significantly different ($P = 0.0086$ for cytoplasmic versus lysosomal counts in normal RPE cells, and $P = 0.0083$ for the same comparison in RPE cells from CNVMs). This appears to be due to the wide separation between the data points for cytoplasmic and lysosomal counts for individual specimens, because there is considerable scatter between the points as a function of age, as indicated by the low *r* values for the best-fitting straight lines. These do not differ significantly from a line of zero slope, suggesting that CRALBP immunogenicity in RPE cells of both normal eyes and CNVMs does not differ significantly with age. Nor does CRALBP immunogenicity differ significantly between normal RPE cells and those in CNVMs, since multivariable logistic regression shows no significant correlations between any of the variables tested (age, sex, cytoplasmic labeling, lysosomal labeling, and hours from death to tissue preparation) and RPE cells from normal subjects compared with those with CNVMs due to ARMD.

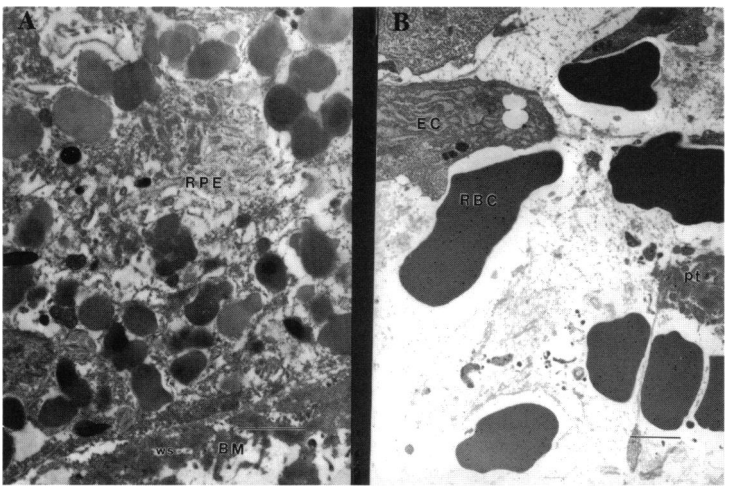


FIGURE 9
Immunostaining for glutathione peroxidase. A, Section through macular RPE of an 83-year-old man with no apparent retinal abnormalities. No labeling is visible in the basal portion of RPE cell (RPE) or of adjacent, thickened Bruch's membrane (BM). Note fragment of wide-spacing collagen (ws). Due to fixation artefact, collagenous portion of Bruch's membrane appears disrupted and basement membrane of RPE is not visible. Sections through other RPE cells from normal eyes, and from CNVMs from subjects with neovascular ARMD, also demonstrated no glutathione peroxidase immunostaining (not shown). B, Section through choriocapillary layer of macular region of same eye. Endothelial cell processes (EC) with evident fenestrations are seen at top of micrograph. Numerous red blood cells (RBC) and single process from platelet (pt) are also visible. Red blood cells, as well as lysosomes in single platelet process, show plentiful immunolabeling for glutathione peroxidase. Calibration bars = 1 $\mu$m.

*Glutathione Peroxidase*

Glutathione peroxidase activity has been reported in RPE cells by several investigators using both biochemical[33,126,128] and immunocytochemical[81] methods. Nevertheless, both immunocytochemical evaluations of human RPE cells (Fig 9) and biochemical determinations on human and bovine RPE cells in vitro (presented below) carried out during the present experiments showed no activity measurable over background. Fig 9A shows the basal portion of the macular RPE layer, and Fig 9B shows red blood cells, a single platelet process, and some endothelial cell processes in the choriocapillaris underlying the macula of a donor eye from an 83-year-old man without evident ocular disease. No immunostain for glutathione peroxidase can be seen in the RPE cell, but copious labeling of the red blood cells as well as of the platelet process is clearly visible.

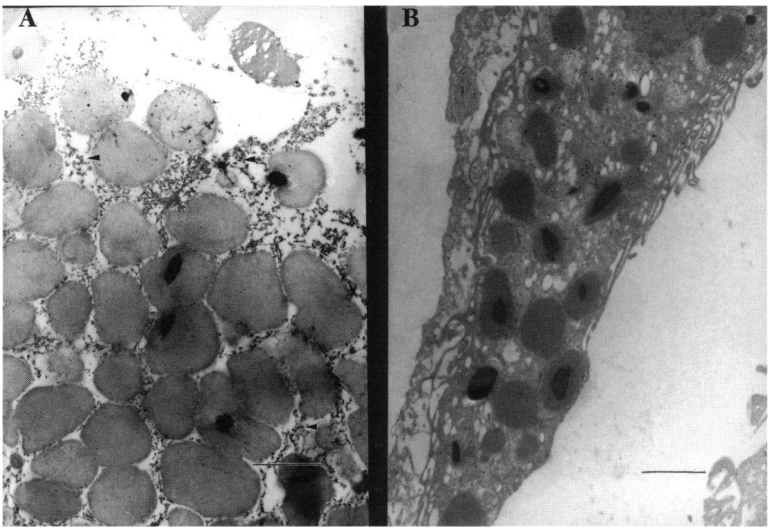


FIGURE 10

Immunostaining for HO-1. A, Section through macular RPE of 54-year-old woman with no apparent retinal abnormalities. B, Section through thinned RPE cell from CNVM from 63-year-old woman with neovascular ARMD. Much of immunogold labeling is present in lysosomes. Arrowheads indicate colloidal gold particles in cytoplasm of Fig 8A, where dense staining of clumped cytoplasmic substance makes them somewhat more difficult to see, especially amidst very plentiful lysosomes in this specimen. Calibration bars = 1 $\mu$m.

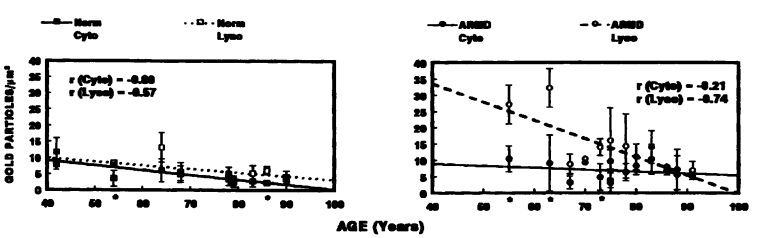


FIGURE 11

Mean (± 1 SD) gold particle counts for specimens immunolabeled with polyclonal antibody against HO-1, plotted as function of age of subject. Left, RPE cells from normal donor eyes. Right, RPE cells from surgical specimens and donor eyes with CNVMs due to ARMD. As in Figs 2, 4, and 6, since both plots represent immunostaining against same primary antibody and plots use identical sets of axes, they may be directly compared. In both plots, filled symbols represent cytoplasmic gold particles and open symbols represent lysosomal gold particles. The $r$ values for best-fitting linear plots (which are illustrated in figures) are shown. Asterisks indicate pairs of values for cytoplasmic and lysosomal labeling of tissue from particular specimen that differ significantly with $P \leq 0.05$ using Student paired $t$ test.

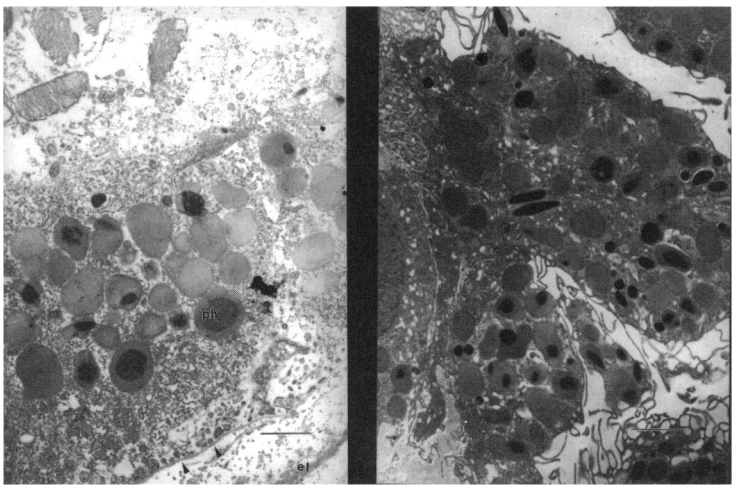


FIGURE 12

Immunostaining for HO-2. A, Section through macular RPE of 72-year-old woman with normal-appearing retina. Photomicrograph was printed at slightly lower magnification than others in this thesis, and one can therefore see distal portion of photoreceptor outer segments (upper left), full-thickness of RPE layer, and portion of Bruch's membrane (lower right). Some of lysosomes contain remnants of phagocytosed photoreceptor outer segments (ph). Portion of layered structure of Bruch's membrane can also be seen, including basement membrane of RPE (arrowheads) and elastic lamina (el), between which is inner layer of fibrillar collagen. B, Section through RPE cells of CNVM from 55-year-old man with ARMD. Copious label is present, especially in lysosomes. This appearance is more prominent in lysosomes from RPE cells in CNVMs from younger subjects that have been immunostained for HO-1 or HO-2. Note also that these RPE cells retain some features of differentiation, notably copious microvilli, but instead of remaining highly oriented at cell apices, these microvilli on adjacent cells appear to be pointing in all directions. Calibration bars = 1 $\mu$m.

*Heme Oxygenase-1 (HO-1), Heme Oxygenase-2 (HO-2)*

Both direct observation of immunoelectron micrographs of RPE cells from normal maculas and from CNVMs from subjects with ARMD (Figs 10 and 12) as well as quantitative measurements of immunolabeling densities in the cytoplasm and lysosomes of these preparations as a function of subject age (Figs 11 and 13) showed several striking features. Unlike any of the other enzymes or cell proteins described in this thesis, both heme oxygenases demonstrated considerably more immunolabeling of lysosomes than of the cell cytoplasm. Statistical comparison of the best-fitting linear plots through the data points yielded highly significant differences in most cases. For HO-1, comparison of the plots for cytoplasm and lysosomes in normal eyes showed no significant difference in slope ($P$ = 0.62) and only a suggestive

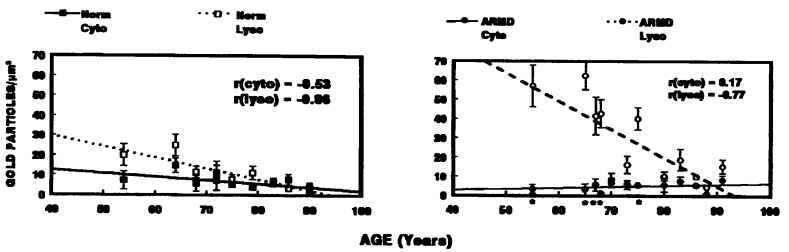


FIGURE 13

Mean (± 1 SD) gold particle counts for specimens immunolabeled with polyclonal antibody against HO-2, plotted as function of age of subject. Left, RPE cells from normal donor eyes. Right, RPE cells from surgical specimens and donor eyes with CNVMs due to ARMD. As in Figs 2, 4, 6, and 9, since both plots represent immunostaining against same primary antibody and plots use identical sets of axes, they may be directly compared. In both plots, filled symbols represent cytoplasmic gold particles and open symbols represent lysosomal gold particles. The $r$ values for best-fitting linear plots (which are illustrated in figures) are shown. Asterisks indicate pairs of values for cytoplasmic and lysosomal labeling of tissue from particular specimen that differ significantly with $P \leq 0.05$ using Student paired $t$-test.

difference in elevation ($P = 0.10$), but the plots for ARMD eyes were highly significantly different for both slope ($P = 0.0087$) and elevation ($P = 0.0021$) (Fig 11). For HO-2, comparison of cytoplasmic and lysosomal gold particle counts in normal eyes showed significant differences in slope ($P = 0.034$) and in elevation ($P = 0.043$), while for ARMD eyes the differences in slope ($P = 0.0078$) and in elevation ($P = 0.0017$) were highly significant (Fig 13). With the exception of the plot of cytoplasmic counts versus age for HO-2 in ARMD eyes ($r = 0.17$, $P = 0.42$), all of the other plots showed negative slopes (ie, decreasing immunogenicity) as a function of subject age, and with one exception, these values varied from borderline to high statistical significance (for HO-1, normal eyes, cytoplasmic counts: $r = -0.80$, $P = 0.009$; lysosomal counts: $r = -0.57$, $P = 0.108$; ARMD eyes, cytoplasmic counts: $r = -0.21$, $P = 0.49$; lysosomal counts: $r = -0.74$, $P = 0.0038$; for HO-2, normal eyes, cytoplasmic counts, $r = -0.536$, $P = 0.137$; lysosomal counts, $r = -0.87$, $P = 0.0024$; ARMD eyes, cytoplasmic counts, $r = 0.17$, $P = 0.62$; lysosomal counts: $r = -0.70$, $P = 0.016$). Multivariable logistic regression using the independent variables described in the sections above with normal and ARMD as the dependent variable showed borderline significance for both age (for HO-1: odds ratio 1.30, 95% confidence interval 0.95-1.79, $P = 0.10$; for HO-2, odds ratio 1.14, 95% confidence interval 0.96-1.35, $P = 0.13$) and for lysosomal counts (for HO-1, odds ratio 2.84, 95% confidence interval 0.92-8.71, $P = 0.067$; for HO-2, odds ratio 1.21, 95% confidence interval 0.95-1.55, $P = 0.12$).

**BIOCHEMISTRY**

Because the human eyes used in these experiments were shipped to the author's laboratory often from great distances and because often considerable time, sometimes in excess of 48 hours, had elapsed before the RPE cells could be isolated for culture (Table I), it was felt necessary to perform some preliminary experiments on bovine RPE cells, which could be obtained fresh (at least, within 2 hours of death) from a local slaughterhouse, in order to learn how the activities of the enzymes studied here varied with time after death in RPE cells isolated from intact bovine eyes, and with time in secondary and tertiary culture for RPE cells assayed after growth in vitro. Figs 14 through 17 show the results of these experiments. In these figures, "fresh" tissue was isolated as described in "Methods and Materials," washed by repeated centrifugation to remove the enzymes used to free the RPE cells from Bruch's membrane, sonicated, and the supernatant assayed for enzyme activity. "Fresh" tissue was assayed either immediately after its arrival in the laboratory ("0 hour") or after refrigeration of the intact globe for 24 hours or 48 hours at 4°C. Alternatively, bovine RPE cells were cultured to confluence, then passaged, and the secondary cultures were assayed after 1, 2, 4, or 8 weeks or, after reaching confluence and passaging again, the tertiary cultures were assayed after 4 or 8 weeks. Fig 14 illustrates the results for total SOD activity and Fig 15 for Mn-SOD, the activity remaining in the presence of 2 mM KCN, which completely inhibits Cu,Zn-SOD. Since activity is measured as the amount of tissue protein required to inhibit reduction of cytochrome C by 50%, the more protein added the less the activity. To plot increasing activity as an increasing function, therefore, the ordinates of Figs 14 and 15 show the reciprocal of the amount of tissue protein added. Both for total SOD and for Mn-SOD activity in bovine RPE cells, there are no significant differences in activity between fresh and cultured cells in secondary or tertiary culture up to 8 weeks, or between fresh cells and cells cultured 24 or 48 hours after death. Since Cu,Zn-SOD represents the difference in activity between these two values, the present results imply that Cu,Zn-SOD activity in the RPE cells also does not vary under the different conditions of cell isolation and preparation used here. These results differ from those described from RPE cells isolated from human eyes by Newsome and associates,[33] who reported twofold to fourfold greater total SOD activity in RPE cells isolated directly from adult or fetal human eyes than in RPE cells from such eyes after culture for periods that were not described.

Catalase activity, however, showed a substantial (and statistically significant) decrease in activity between RPE cells isolated directly from the intact bovine globe, even as long as 48 hours after death, and bovine RPE cells in culture (Fig 16). Cells either assayed immediately after arrival of the eyes in the laboratory, or placed in culture immediately after arrival,

TABLE I: SUMMARY DATA ON DONOR EYES AND CHOROIDAL NEOVASCULAR MEMBRANES USED IN THIS STUDY

| AGE (YR) | SEX | RACE | CAUSE OF DEATH | TIME FROM DEATH TO RECEIPT OF SPECIMEN IN LABORATORY (HR) |
|---|---|---|---|---|
| **WHOLE EYES** | | | | |
| **NORMAL IMMUNOCYTOCHEMISTRY** | | | | |
| 42 | F | W | Head and chest trauma in motor vehicle accident | 43 |
| 48 | F | W | Pneumonia, dementia | 42 |
| 54 | F | W | Renal failure, renal-cell carcinoma | 41 |
| 60* | F | W | Myocardial infarction | 40 |
| 64 | M | W | Cerebrovascular accident; renal failure | 28 |
| 65 | F | W | Congestive heart failure | 32 |
| 68 | M | W | Sepsis, multisystem failure | 40 |
| 72* | F | W | Respiratory failure secondary to pulmonary metastases, renal-cell carcinoma | 25 |
| 74 | M | W | Not given | 38 |
| 75 | F | W | Cerebrovascular accident | 32 |
| 76 | F | W | Cerebrovascular accident | 35 |
| 78 | F | W | Renal failure | 37 |
| 79 | M | W | Cardiomyopathy, ventricular fibrillation | 39 |
| 83 | M | W | Intracranial hemorrhage | 46 |
| 85 | F | W | Myocardial infarction | 47 |
| 86 | M | W | Cardiomyopathy | 36 |
| 90 | F | W | Alzheimer's disease, respiratory failure | 27 |
| 92 | F | W | Myocardial infarction | 32 |
| 94 | F | W | Bowel infarction | 41 |
| **CELL CULTURE** | | | | |
| 40 | M | W | Accident | 48 |
| 45 | F | B | Cardiac arrest | 34 |
| 60 | F | W | Coronary artery disease (diabetes) | 30 |
| 71 | M | W | Not given | 26 |
| 76 | M | W | Cerebrovascular accident (insulin-dependent diabetes) | 30 |
| 77 | M | W | Intracranial hemorrhage | 24 |
| 83 | M | W | Respiratory arrest | 31 |
| 88 | F | W | Not given (non-insulin-dependent diabetes) | 32 |
| 88 | F | W | Pneumonia | 21 |

TABLE I (CONTINUED): SUMMARY DATA ON DONOR EYES AND CHOROIDAL NEOVASCULAR MEMBRANES USED IN THIS STUDY

| AGE (YR) | SEX | RACE | CAUSE OF DEATH | TIME FROM DEATH TO RECEIPT OF SPECIMEN IN LABORATORY (HR) |
|---|---|---|---|---|
| **MACULAR DEGENERATION** | | | | |
| **IMMUNOCYTOCHEMISTRY** | | | | |
| 75 | F | W | Coronary artery disease, renal failure | 45 |
| 91 | F | W | Respiratory arrest (glaucoma, diabetes) | 41 |
| **CELL CULTURE** | | | | |
| 76 | F | W | Not given | 35 |
| 83 | F | W | Intracranial hemorrhage | 44 |
| 84 | F | W | Respiratory failure | 30 |
| 88 | F | W | Renal failure | 45 |
| 88 | F | W | Pulmonary embolus | 42 |
| 92 | F | W | Myocardial infarction | 36 |
| **SURGICAL SPECIMENS (CHOROIDAL NEOVASCULAR MEMBRANES)** | | | | |
| 55 | M | B | | |
| 63 | F | W | | |
| 65 | F | W | | |
| 67 | M | W | | |
| 68 | M | W | | |
| 70 | F | W | | |
| 73 | F | W | | |
| 75 | M | B | | |
| 76 | M | W | | |
| 78 | M | W | | |
| 79 | F | W | | |
| 80 | F | W | | |
| 83 | F | W | | |
| 85 | F | W | | |
| 86 | M | W | | |
| 87 | F | W | | |
| 88 | F | W | | |
| 91 | F | W | | |
| 92 | F | W | | |

*The fellow eye of these cases was also used for cell culture.

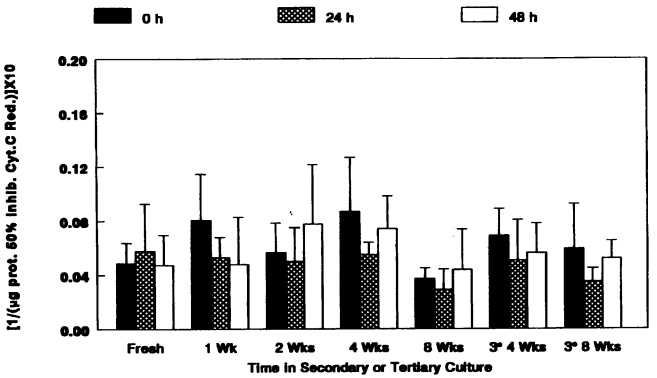


FIGURE 14

Total SOD activity in bovine RPE cells. "Fresh" cells were assayed directly after removal from intact eyes either immediately upon arrival in laboratory or after incubating intact globe in refrigerator at 4°C for 24 or 48 hours. Cells were also assayed after secondary or tertiary culture for the times shown. Bars show mean values +1 SD for determinations made on 3 separate cultures. Although a few pairs of values differed significantly from one another with $P \leq 0.05$ by Student $t$-test, there were no significant differences demonstrable by any multiple range test and no consistent differences between fresh and cultured cells, or cells at different passage levels. In this figure and in Figs 13 through 15, negative standard deviations are not shown, but are equal in magnitude and opposite in sign to positive standard deviations that are depicted.

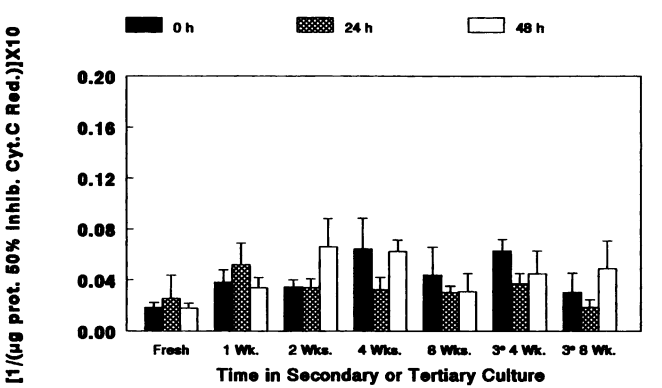


FIGURE 15

Mn-SOD activity in fresh or cultured bovine RPE cells. Mn-SOD is activity remaining in presence of 2 mM KCN, which completely inhibits Cu,Zn-SOD. Other conditions are as described for Fig 12. Plots show mean values +1 SD for determinations made on 3 separate cultures. Values for -1 SD are of equal magnitude in opposite direction. Although some individual pairs of values differed from one another with $P \leq 0.05$ by Student $t$-test, there were no significant differences using any multiple range test and no consistent differences between fresh and cultured cells, or cells at different passage levels.

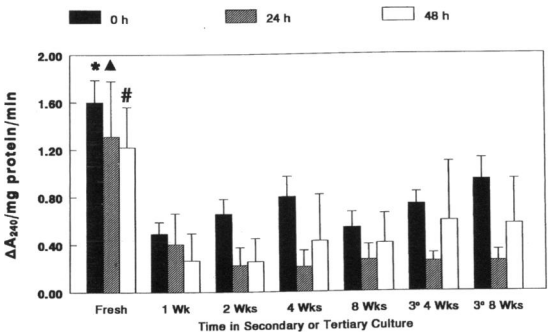


FIGURE 16

Catalase activity in fresh or cultured bovine RPE cells. Other conditions are as described for Fig 12. Plots show mean values +1 SD for determinations made on 3 separate cultures. Values for -1 SD are of equal magnitude in opposite direction. * = Differs from values from cultured cells at all time periods, $P \leq 0.01$. ▲ = Differs from values from cultured cells at all time periods, $P \leq 0.05$. # = Differs from values from secondary cultures at 1 and 2 weeks, $P \leq 0.01$, but does not differ significantly from values at later time periods or in tertiary culture. Statistical calculations used Newman-Keuls multiple range test. Although some individual pairs of values for cultures at different passage levels or time periods differed from one another with $P \leq 0.05$ by Student $t$ test, there were no significant differences using any multiple-range test other than those specifically marked in this plot.

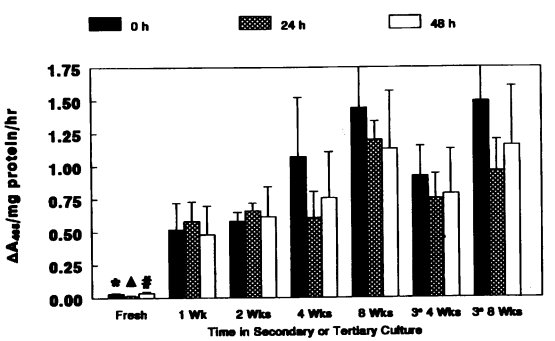


FIGURE 17

HO-1 activity in fresh or cultured bovine RPE cells. Activity was determined after exposing cultured cells to 10 $\mu$M sodium arsenite in 1% fetal bovine serum overnight. Activity in absence of arsenite stimulation presumably represents HO-2. Although immunocytochemical studies showed considerable HO-2 antigen, enzyme assay under these conditions showed little activity and therefore plots of HO-2 activity in bovine RPE cells are not shown. Other conditions are as described for Fig 12. Plots show mean values +1 SD for determinations made on 3 separate cultures. Values for -1SD are of equal magnitude in the opposite direction. * = Differs from values from cultured cells at all time periods, $P \leq 0.01$. ▲ = Differs from values from cultured cells at all time periods, $P \leq 0.003$. # = Differs from values from cultured cells at all time periods, $P \leq 0.026$. Statistical calculations used Newman-Keuls multiple-range test. Although some individual pairs of values for cultures at different passage levels or time periods differed from one another with $P \leq 0.05$ by Student $t$-test, there were no significant differences other than those specifically marked in this plot using any multiple-

always demonstrated more activity than cells assayed or cultured after a 24- or 48-hour delay. The differences were statistically significant in some instances. These results comparing catalase activity in freshly isolated bovine RPE cells with cultured cells are in agreement with results reported by Newsome and associates.[33] Activity in cells in either secondary or tertiary culture in the present experiments did not change significantly with time, at least up to 8 weeks.

HO-1 activity in bovine RPE cells, however, showed a statistically highly significant increase with time in culture by comparison with activi-

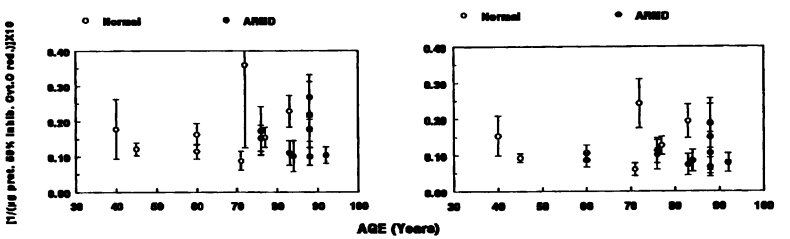


FIGURE 18

Left, Total SOD activity in second passage cultures of human RPE cells from donor eyes as function of age. Right, Mn-SOD activity in second passage cultures of human RPE cells from donor eyes as function of age. Cu,Zn-SOD activity may be calculated as difference between pairs of data points for total and Mn-SOD activity for each individual specimen. In both plots, values shown are mean ± 1 SD of 3 separate determinations. Filled symbols indicate cells from individuals with ARMD.

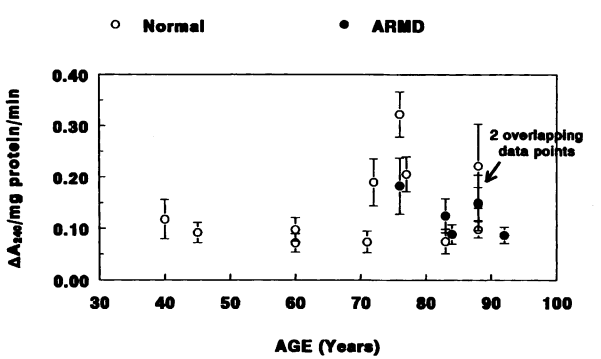


FIGURE 19

Catalase activity in second passage cultures of human RPE cells from donor eyes as a function of age. Values shown are the mean ± 1 SD of 3 separate determinations. Filled symbols indicate cells from individuals with ARMD.

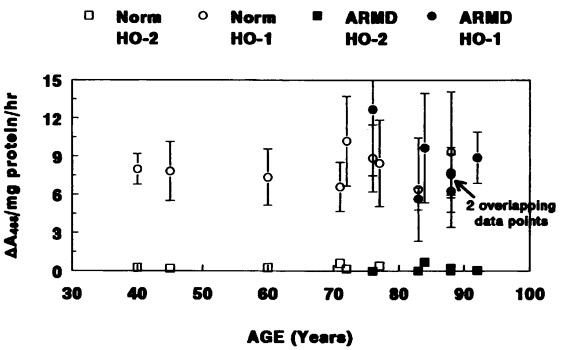


FIGURE 20

Heme oxygenase activity in second passage cultures of human RPE cells from donor eyes as function of age. HO-1 was determined in cultures that had been exposed to arsenite overnight as described in text. There is virtually no detectable HO-2 activity (not stimulated by arsenite) in these cultures. Values shown are mean ± 1 SD of 3 separate determinations. Filled symbols indicate cells from individuals with ARMD.

ty in "fresh" tissue. Activity in cells either assayed or placed in culture immediately after arrival in the laboratory was usually greater, though not always significantly so, than activity in cells from eyes that were prepared after a 24- to 48-hour delay (Fig 17). HO-2 (non-arsenite-stimulated) activity in these cultures was extremely low, barely above the threshold for measurement in all cultures of human RPE cells assayed, and is not shown here.

Activity of total SOD and the KCN-resistant fraction (Mn-SOD), of catalase, and of HO-1 and HO-2 measured in cultured human RPE cells at the second passage showed no significant trend with age of the subject (Figs. 18 through 20). There was considerable scatter in the data points, and the best fit for each curve was a straight line, but the *r* values were low and none of the plots differed significantly from a plot of zero slope (ie, showing neither an increase nor a decrease in activity with increasing age). Of particular interest is the data for heme oxygenase enzyme activity shown in Fig 20. Figs 12 and 13 show immunocytochemical data indicating a considerable amount of HO-2 antigen in RPE cells, albeit primarily in lysosomes, yet determinations of HO-2 enzyme activity in this tissue showed almost none.

In the present experiments, no detectable glutathione peroxidase activity was found in cultured bovine or human RPE cells when assays were carried out at pH 7.0, 7.2, or 8.0. Different pHs were used because of the high background activity in the reaction mixture in the absence of enzyme at pH 8.0, said to be the pH optimum for the native erythrocyte

enzyme. (Sigma, which supplies glutathione peroxidase commercially, performs its assays at pH 7.0 for this reason.) Because no enzyme activity was found in cultured RPE cells, an assay of rat liver homogenates, containing erythrocytes, was carried out. This yielded activities at pH 7.2 of 0.21 (± 0.05 SD) absorbance units (AU)/min/mg protein using cumene hydroperoxide as substrate, and 0.06 (± 0.02 SD) AU/min/mg protein using $H_2O_2$. These data were derived from 5 assays using each substrate.

## DISCUSSION

This study has attempted to evaluate the hypothesis that the primary event in the pathogenesis of ARMD occurs in the macular RPE cells and involves alterations in the quantity and/or activity of one or more "oxidative protector" enzymes. As previously noted, the recent finding[35] that a heterozygous mutation in the gene for the ABCR protein of the photoreceptor outer segments is present in 16% of a series of ARMD patients does not exclude the possibility that this genetic abnormality, and several others, might ultimately disrupt the metabolism of RPE cells (for example, by altering the phagocytosis and degradation of the abnormal outer segments within the RPE to lead to higher levels of potentially toxic oxidative end products) to produce an increase in oxidative damage. Thus, the hypothesis might better be restated that, *following one of several possible initiating lesions, the primary event in the final common pathway leading to the abnormalities of ophthalmoscopic anatomy and visual function in ARMD occurs in the macular RPE cells and involves alterations in the quantity and/or activity of one or more "oxidative protector" enzymes.*

This hypothesis was tested by evaluating levels of several of these enzyme antigens in macular RPE cells from subjects with no visible retinal lesions by comparison with antigen levels in subjects with CNVMs resulting from ARMD. The first conclusion to be reached from these results is that Cu, Zn-SOD, catalase, and glutathione peroxidase in the RPE do not appear to be closely related to the pathogenesis of ARMD. In both cytoplasm and lysosomes of macular RPE cells from both normal and ARMD eyes, Cu, Zn-SOD antigen levels tended to increase with age. However, in comparing antigen levels in ARMD versus normal macular RPE cells, only cytoplasmic Cu, Zn-SOD showed borderline significance with $P = 0.07$, but with an odds ratio of 0.92, near unity. This study compared RPE cells from normal eyes with RPE cells from eyes with the most severe form of ARMD, in which biochemical differences from normal tissues related to the disease state would be expected to be maximal. Therefore, despite its borderline statistical significance, the biological importance of this small difference between normal and diseased eyes would seem to be lacking. In the only study comparable to the present

one, Liles and associates[94] reported total SOD activity in macular and peripheral RPE cells of subjects with normal eyes and those with early ARMD. They found a slight but statistically nonsignificant increase in activity in ARMD subjects by comparison with normals, and no change with age.

Similar to the study of Liles and associates, who also assayed catalase enzymatic activity and found a decrease with increasing age in both normal eyes and those with (apparently early, on the basis of the description given) ARMD,[94] the present results show a decrease in catalase antigenicity with age in macular RPE cells from both control and ARMD eyes, but unlike these investigators, who found significantly decreased catalase activity in both macular and peripheral RPE cells in subjects with ARMD by comparison with normals, the present experiments find no difference in antigen levels between the two conditions.

The experiments on glutathione peroxidase in RPE cells reported here demonstrated no enzyme antigen or measurable activity above background, while previous investigators have reported that glutathione peroxidase activity is present in the RPE[33,81,126,128] and in other ocular tissues, including the iris.[81] In contrast to these reports but similar to the present results, Marshall[82] has recently reported the absence of glutathione peroxidase antigenicity in human iris stroma and pigment epithelium studied by electron microscopic immunocytochemistry using colloidal gold labeled second antibodies. In Marshall's experiments, glutathione peroxidase immunoreactivity was strongly present in red blood cells, confirming the validity of his immunocytochemical technique. An explanation for these differences is speculative. Atalla and colleagues[81] used an antibody that they prepared themselves for their immunocytochemical studies, while the present experiments and those of Marshall employed commercially available polyclonal antibodies. The positive biochemical results for glutathione peroxidase in RPE and other ocular cells described by previous investigators may not have considered sufficiently the high "background" oxidation of NADPH that occurs simply with the admixture of GSH and cumene hydroperoxide or $H_2O_2$ with NADPH, or of these reagents and GSSG reductase, even without the addition of the tissue sonicate being assayed. In consideration of the possibility that contaminants in some of these reagents might produce this "background" reaction, several lots of cumene hydroperoxide from Sigma (the only commercial supplier) were tested in the author's laboratory, as well as several lots of GSSG reductase from Sigma and from ICN Pharmaceuticals (Costa Mesa, CA) without reduction of the background. Enzyme activity of the RPE cell sonicates could not be detected above the high background, while the much higher glutathione peroxidase activity of rat liver sonicates was easily demonstrable.

The most striking positive results of the present experiments are the measurements of heme oxygenase antigenicity in RPE cells. Lysosomal counts for HO-1 showed a difference between ARMD and normal subjects of borderline significance ($P = 0.067$), but also with a very large odds ratio, 2.84, and a 95% confidence interval of 0.92 to 8.71. The difference between ARMD and normal eyes for lysosomal HO-2 levels was also of borderline significance, $P = 0.12$, and with an odds ratio of 1.21 and 95% confidence interval of 0.95 to 1.55. While these values do not achieve the $P \leq 0.05$ level required for statistical significance, they are substantially closer to significance than values for the other RPE cell proteins studied here, save for cytoplasmic Cu, Zn-SOD, whose odds ratio, as noted, was so close to unity as to render questionable the biological significance of this very small $P$ value. In addition, the heme oxygenases demonstrated the most marked changes in antigen level with age of any of the molecules studied, with generally large decrements in concentration as a function of age indicated by large negative $r$ values that were in several cases highly significantly different from lines of zero slope. Of particular interest are the substantially increased levels of HO-1 and HO-2 antigens in RPE cell lysosomes by comparison with cytoplasmic antigen. The differences between the slopes and elevations of the curves of antigen level versus age (Figs 11 and 13) of lysosomal versus cytoplasmic labeling for these two enzymes were statistically highly significant This feature was not characteristic of any of the other proteins studied here. By contrast, Cu, Zn-SOD showed no significant differences in the slopes of the curves for cytoplasmic and lysosomal labeling versus age, with differences in the curves for elevation of borderline significance and with the cytoplasmic labeling per unit area slightly higher than that of the lysosomes. Catalase labeling also showed no significant differences in the slopes of the curves for cytoplamic or for lysosomal labeling with age, though the cytoplasmic labeling showed a significant difference in elevation with lysosomal labeling in the normal tissues, but not that for eyes with neovascular ARMD (Fig 6). Also for this enzyme, cytoplasmic labeling per $mm^2$ was somewhat greater than that for lysosomal labeling. Finally, CRALBP showed the most marked increment in the elevation of the curve for cytoplasmic labeling per unit area by comparison with labeling of lysosomes (Fig 8). In all of these cases, cytoplasmic labeling exceeded that of the lysosomes, indicating that the reverse behavior of the immunolabeling for HO-1 and HO-2 is likely to have biological importance.

Explaining the biological meaning of this finding at the present time, however, requires speculation. Lysosomes are organelles whose major function is the enzymatic degradation of cellular components. Presumably, therefore, cellular organelles and molecules that are found within lysosomes have lost much or all of their biological activity. One

must determine whether the presence of substantial immunolabeling within lysosomes for a molecule that normally has highly specific biological activity is artefactual or real. That intralysosomal immunolabeling for HO-1 and HO-2 is not artefactual is suggested, first, by the fact that such labeling was not present in control sections in which nonimmune IgG was substituted for the specific primary antibody. The labeling therefore does not represent nonspecific binding of IgG to lysosomes. Second, the relative proportion of cytoplasmic to lysosomal labeling differs widely among the various antigens investigated in the present study. Since all of the antibodies used here were IgGs, it is hard to imagine that "nonspecific" labeling could vary so greatly from one antibody of the same class to another. Third, greater immunolabeling of lysosomes than of cytoplasm has also been demonstrated in studies of acidic and basic fibroblast growth factors in cultured bovine retinal microvascular endothelial cells[129] and in RPE cells from intact eyes of rats following experimental laser treatment and in CNVMs removed surgically from humans with neovascular ARMD.[130,131] These observations provide additional evidence that the intense immunolabeling of lysosomes with antibodies to specific proteins represents a biologically valid phenomenon. That antigen within lysosomes binds to antibody means that, whatever degradation of the antigen molecule has taken place, its antigenic site(s) remain intact. The present results appear to indicate that there are considerable differences in the lysosomal uptake of the protein molecules under investigation. Alternatively, these results may have the trivial explanation that, while lysosomal uptake of all of the molecules is approximately equal, the antigenic sites on some of these molecules are more resistant to lysosomal enzyme degradation. A point against this argument is that the present experiments were carried out with polyclonal antibodies, which are reactive against several antigenic sites, at least some of which are likely to be more resistant to lysosomal degradation. This should preserve the antigenicity of these molecules even within lysosomes better than had monoclonal antibodies, which only react with a single epitope on the antigen molecule, been used.

As regards the biological importance of these observations, the heme oxygenases have received considerable recent attention because of the relationship of HO-1 induction to oxidative stress, but there have been until the present results no published results dealing with the possible relationship of heme oxygenase activity to ARMD. It has been emphasized that HO-2 is a constitutive enzyme, while HO-1 is induced by oxidative stress, although the present quantitative immunocytochemical results show similar but not identical behavior of the two enzymes in macular RPE cells from human eyes as a function of age and of neovascular ARMD. (That HO-1 but not HO-2 enzyme activity increased substantially in cultured bovine RPE cells by comparison with cells freshly isolated

from intact eyes suggests that culture itself induces oxidative stress.) Although the action of such a powerful "oxidative protector" enzyme is thought to be beneficial to cells undergoing oxidative stress, Kutty and associates[53] have pointed out that HO-1 induction may also have deleterious effects, including the formation of chelatable iron from the breakdown of heme, and the production of intracellular carbon monoxide. Therefore, although the induction, and action, of HO-1 in the presence of oxidative stress may have a protective function, its potentially harmful effects may be a reason for the cell to dispose of heme oxygenase proteins rapidly when their production becomes excessive. The results of the present study, therefore, suggest the following conclusions: A variety of initial lesions in the outer layers of the neural retina or in the RPE can result in ARMD. These lesions produce severe oxidative stress in macular RPE cells. HO-1 (and possibly HO-2) is (are) induced by the cell as a response to the stress, but because these enzymes are not normally present in RPE cells at such high levels, and because high enzyme levels may also have adverse effects, the cell attempts to dispose of superfluous enzyme protein through lysosomal degradation to minimize tissue damage. Because of the possible damaging effects of the heme oxygenases, and because intralysosomal HO-1 immunoreactivity is significantly greater in RPE cells from subjects with ARMD than in RPE cells from control subjects, an excess of these enzymes in RPE cells as a result of oxidative stress may be a principal cause of the later lesions of ARMD. Against this argument, however, is the observation that the increased HO-1 immunoreactivity is much greater in younger subjects with ARMD than in older subjects with the disease, whereas both the incidence and prevalence of ARMD and of its neovascular form increase sharply with greater age. Therefore, a more likely possibility is that the increased induction of HO-1 in cells from younger individuals by comparison with older subjects simply indicates a greater protective effect in younger individuals, which explains why they are better able to withstand the initial pathogenetic event. That HO-1 induction is quantitatively greater in individuals with ARMD than in control subjects reflects the greater magnitude of this initial stressful event in individuals who develop the disease. What is somewhat surprising is the observation that HO-2 immunoreactivity in lysosomes shows a similar response, though of smaller magnitude, in individuals with ARMD by comparison with controls, since HO-2 is thought to be a constitutive, rather than an inducible, enzyme. At present, there is no evident explanation for this finding.

This study, then, suggests that at least the neovascular form of ARMD involves oxidative stress in its pathogenesis, but the nature of the stressful event is unknown. As noted in the discussion of the ABCR gene and ARMD, there may be several initiating events that lead to this final path-

way in which oxidative stress is critical. This implies that appropriate therapy that can combat the oxidative stress may slow the development of, or prevent entirely, choroidal neovascularization in this disease. Whether oxidative stress is involved in the initiation of the "dry," or atrophic form of ARMD, or of the initial lesions of the disease, including druse and Bruch's membrane changes, cannot be answered from the data presented here because ocular tissue containing these early lesions was not obtainable during the course of this study. In fact, the reason that this study was possible was the fact that surgical excision of CNVMs in patients with ARMD has been considered a therapeutic possibility over the last few years, leading to the availability of such tissues with late stages of the disease for laboratory analysis. However, the negative results regarding Cu, Zn-SOD, catalase, and glutathione peroxidase in comparing tissue from whole eyes and surgical specimens with far advanced, neovascular ARMD clearly indicates that these enzymes cannot be responsible for initiating the early forms of this disease, and the highly suggestive results with the heme oxygenases leaves open the possibility that oxidative stress is involved in the pathogenesis of early ARMD. Eyes with lesions of any form of ARMD have been very infrequent in donor material obtainable from local eye banks or national organizations like NDRI in the experience of this author. Surgical specimens containing macular RPE cells from patients with early ARMD would not be obtainable under any but the most unusual circumstances, since there is no ethically acceptable diagnostic or therapeutic reason why such tissue would be excised from the eyes of living human subjects at the present time. There has been only one published study[94] in which enzyme analyses of RPE cells from subjects with apparently early forms of ARMD were compared with RPE cells from normal eyes. Other reports have only described the relationship of age to various enzyme activities in the human retina or RPE, but have not included eyes with ARMD.[34,64,132,133]

Indeed, in considering the methodology to be used in the present experiments, measurement of enzyme activity appeared less likely to yield useful results because it seemed more probable that enzyme activity would vary substantially in the often many hours that must necessarily elapse from the time of death to the receipt of a donor eye in the laboratory than that antigenically detectable enzyme protein would undergo similar variation. In fact, significant variation of activity with time after death up to 48 h did not seem to be a major occurrence for the enzymes evaluated here. However, because of the extremely small size of the surgically excised CNVM specimens, the measurement of enzyme activity in RPE cells from these tissues could not be carried out. For the present study, therefore, quantitative immunocytochemistry appeared more suitable, and the results obtained suggested that this method was better able to dis-

tinguish consistent variations with age as well as with localization of enzyme proteins to cellular organelles. Enzyme assays comparing fresh to cultured bovine RPE cells showed substantial variations in activity between freshly isolated cells and cells grown in culture. For some enzymes there was no significant change in activity between freshly isolated or cultured RPE cells, or with time in culture (total SOD or Mn-SOD, Figs 14 and 15). Other enzymes showed a marked decrease in activity between fresh and cultured RPE cells (catalase, Fig 16), while the activity of still others increased markedly in cultured RPE cells by comparison with freshly isolated ones (HO-1, Fig 17). If the same enzymes in human RPE cells behave similarly to these observations on bovine RPE cells in freshly isolated cells and in culture, then there may be no significant differences in the activities of the several enzymes investigated as a function either of age or of the presence of advanced ARMD (Figs 18 through 20). However, these data on enzyme activity in cultured human RPE cells are puzzling both because they are at some variance with previously published results[94] and because of their differences from the quantitative immunocytochemical results described here.

Finally, a brief consideration of the methodology used in these experiments is worth considering. There are 3 possible methods that can be used in a study of this type. Quantitative molecular methods, such as quantitative northern blotting or quantitative reverse transcriptase-polymerase chain reaction, cannot be used with donor eye tissue because during the time elapsed since death, enzymatic degradation of the nucleic acids occurs, rendering the results of such studies of questionable value. Direct assay of the enzymes under investigation is a possible method, providing sufficient tissue is available,[94] but could not be used with the surgical specimens described in the present work because of their very small size. The assays described in this thesis showed that activity of the enzymes studied here was not significantly diminished when the time elapsed from death to processing of the tissues was as long as 48 hours, but there was considerable variation in activities from human RPE cells following culture and no consistent variation with age.

Electron microscopic immunocytochemistry for quantitative determination of the amount of a particular antigen present is theoretically sound, but several precautions are required.[119,120,134] Similar precautions have been stated for immunocytochemistry in general, even when it is not being used in a quantitative fashion.[135] Immunocytochemical quantitation is a valid technique if the tissues being compared are fixed, embedded, sectioned, and stained in precisely the same way, and if the same batches of reagents, including antibodies, are used for each specimen. All of these precautions were carefully observed in the present study; the only difference in preparation between the different groups of specimens was that most of the tis-

sues with disciform ARMD were removed surgically and were fixed immediately in the operating room, while all of the control specimens and only some of the ARMD specimens were obtained postmortem, with considerably greater elapsed time between death and fixation of the eye. Several findings strongly suggest that this difference in time between death and preparation of the tissue in the laboratory had no important bearing on the results reported here. First, the enzyme activity studies described here (Figs 14 through 17) show no significant difference in activity of the enzymes under investigation between bovine RPE cells isolated 2 hours after the death of the animal and those incubated in the refrigerator at 4°C for up to 48 hours, the maximum time between death and arrival of human donor eyes in the author's laboratory. Although quantitative immunocytochemistry is a different technique than direct enzyme assay, it is safe to assume that tissues with comparable activities of a given enzyme possess comparable amounts of enzyme protein (antigen). The converse may not be true, however, since a protein may retain its antigenicity even though its biological (here, enzymatic) activity has been lost. Second, multivariable logistic regressions for each of the enzymes, and for CRALBP, studied by quantitative immunocytochemistry, showed no significant correlation between time from death or surgical excision of the tissue until its processing in the laboratory, and antigenicity. (For the surgically excised CNVMs, this time was arbitrarily assigned the value 1 hour.)

This study has brought us, perhaps, a bit closer to the understanding of ARMD, but it is also clear how far we have yet to go to understand its pathogenesis completely, to treat it effectively, and still more important, to prevent its occurrence. As the introduction to this thesis has suggested, ARMD is a disease that has been well known to ophthalmologists for almost a century and a half, but it has only been within the past 30 years that we have begun to comprehend its development at the cellular level, to learn details of its epidemiology, and to begin to develop some methods of treatment of even limited success. Recent advances in laboratory technology are now beginning to permit the study of ARMD at the level of molecules. Yet a full knowledge of this common and devastating ocular disease will continue to require extensive exploration. In "The Four Quartets," the last poem of T.S. Eliot, written more than 20 years before his death, he eloquently expressed the nature of scientific investigation and its relation to the understanding of things that are familiar in our experience.

> *"We shall not cease from exploration*
> *And the end of all our exploring*
> *Will be to arrive where we started*
> *And know the place for the first time."*
> —TS Eliot, "Little Gidding"
> from "The Four Quartets," 1942

ARMD is certainly a familiar disease, but it remains one whose nature we continue to explore in the hope that we will eventually know it well enough to alleviate its devastation.

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