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ORIGINAL ARTICLE

Light Filtering in a Retinal Pigment Epithelial Cell Culture Model

Jilin Zhou* and Janet R. Sparrow†

ABSTRACT

Purpose. We tested for protection of blue light-exposed A2E-containing retinal pigment epithelial (RPE) from damage through the implementation of polycarbonate filters containing varying levels of a pigment that absorbs short wavelength light.

Methods. Human adult RPE cells (ARPE-19) that had accumulated synthesized A2E were exposed to either a light line delivered from a tungsten halogen source (430 ± 20 nm; 8 mW/cm^2) or to the entire area of a 35 mm dish (1 mW/cm^2). Blue-light absorbing polycarbonate filters (2.5 × 4 cm) containing varying levels of short-wavelength light absorbing pigment (1.0, 1.9, 3.8, 7.5, 15, and 35 ppm) or no dye (PC) were placed in the light path. Cytotoxicity was measured by the 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric microtiter assay (Roche Diagnostics Corporation, Indianapolis, IN) or by fluorescent staining of non-viable cells.

Results. When filters containing blue-light absorbing dye were placed in the light path, protection of 430 nm irradiated A2E-laden RPE was observed. The extent of protection was dependent on the concentration of the dye. By MTT assay and fluorescence labeling, statistically significant differences ($p < 0.05$) between irradiation in the absence of a filter and irradiation in the presence of a filter were observed.

Conclusions. The series of filters tested in this work provided protection against blue light damage in a culture model. (Optom Vis Sci 2011;88:1–●●●)

Key Words: retinal pigment epithelium, RPE, blue light filter, cell viability

The finding that photic damage could be detected at light levels that were too low to involve thermal mechanisms,[1] alerted investigators to the propensity for light to damage retina. Since then light damage has been sorted into two broad classes based on exposure parameters.[2] In particular, exposures of long duration and low-intensity light (Noell-type damage; type I)[2] result in damage manifested in photoreceptor cells. Under these exposure conditions, the action spectrum for sensitivity to threshold damage that peaks at ~500 nm also corresponds to the absorption spectrum of visual pigment rhodopsin,[1,3] suggesting that the initial events in photoreceptor cell injury involve absorption of photons by the 11-cis-retinal chromophore of visual pigment. However, when relatively high-intensity light is combined with short exposure times (Ham damage; type I),[2,4] cellular damage increases at shorter wavelengths of light, with damage peaking at ~440 nm.[5–9] This type of damage has come to be known as "blue light damage." The site of origin of blue light damage in primates appears to be both photoreceptor outer segments and retinal pigment epithelial (RPE) cells, although the greatest damage is reported in RPE cells.[5,10–14] Photooxidative mechanisms are clearly involved because antioxidants can protect against photic damage and increased oxygen availability lowers the threshold.[10]

There is now considerable evidence that fluorescent pigments that accumulate in RPE cells as lipofuscin are responsible, at least in part, for blue light damage to RPE cells. The lipofuscin that accumulates in RPE cells with age is stored within the membrane bound organelles of the lysosomal compartment of the cell (lipofuscin granules). Significantly, native lipofuscin in the RPE exhibits an excitation spectrum that peaks at ~460 to 475 nm and an emission maximum at 590 to 620 nm.[15] Concerted efforts over the past few years have succeeded in identifying some of the constituents of RPE lipofuscin including the bisretinoid compounds A2E, iso-A2E, other cis-isomers of A2E, unconjugated all-trans-retinal dimer (atRAL dimer) and all-trans-retinal dimer conjugated to phosphatidylethanolamine (all-trans-retinal dimer- phosphatidylethanolamine, atRAL di-PE) and ethanolamine (atRAL dimer-ethanolamine, atRAL di-E).[16–20] For all these lipofuscin pigments, the carbon-carbon double bonds residing along the side-arms and within the head group provide the extended conjugation system that allows for absorption and excitation by wavelengths in the visible range of the spectrum.

*MD
†PhD
Department of Ophthalmology (JZ, JRS), and Department of Pathology and Cell Biology (JRS), Columbia University, New York, New York.

Studies have shown that the accumulation of A2E by cultured RPE confers a susceptibility for blue light-induced cell death,[21–23] which is not manifested by cells devoid of A2E.[21,22] As demonstrated by the presence of TUNEL-positive cells and by Annexin V-labeling of externally exposed phosphatidylserine, it is also clear that the cells undergo an apoptotic form of cell death.[21,22] The apoptosis program, which is initiated by blue light illumination of A2E involves the activation of caspase-3,[22] a downstream cysteine-dependent protease that cleaves manifold cellular substrates and brings the cell to its demise. Moreover, A2E-loaded blue light-exposed RPE cells can be rescued from cell death by transfection with Bcl-2,[22] an anti-apoptotic protein situated in the outer mitochondrial membrane. The greater damage evoked by blue light when compared with green light reflects a wavelength-dependency that is consistent with the excitation spectra of A2E.[21] Moreover, evidence indicates that the generation of oxygen reactive species on photoexcitation of A2E is integral to the death of the cells. For instance, an enhancer (D_2O) and quenchers (histidine, DABCO, azide) of singlet oxygen modulate the incidence of non-viable A2E-laden RPE after blue light illumination.[24] Significantly, A2E also undergoes photochemical changes that involve the insertion of oxygens at carbon-carbon double bonds to generate highly reactive oxo-A2E compounds.[24,25] These novel derivatives of A2E, along with singlet oxygen and perhaps other reactive oxygen species, may be responsible for damaging the cell. Although A2E has been the most intensively studied of the bisretinoid compounds of RPE lipofuscin, unconjugated atRAL dimer is a more efficient generator of singlet oxygen than A2E and the all-trans-retinal dimer series is more readily photooxidized.[26]

Efforts have also been made to develop therapeutic strategies for limiting the impact of lipofuscin accumulation in RPE. For instance, some approaches such as vector-based gene therapies[27,28] and the use of small molecule compounds aim to reduce the formation of RPE lipofuscin pigments.[29–32] However, antioxidants such as vitamin E and C can neutralize the photooxidative processes initiated by these bisretinoid compounds.[24,33] As an even simpler approach, it has been suggested that the biosynthesis of bisretinoid lipofuscin compounds and the lipofuscin-associated photo-oxidative processes can be reduced by wearing lenses with appropriate transmission properties.[34–36]

Here, we tested the effectiveness of protecting blue light-exposed A2E-containing RPE from damage through the implementation of blue absorbing polycarbonate filters containing varying levels of a blue absorbing pigment (High Performance Optics, Roanoke, Virginia). This technology is the first tested in an ophthalmic lens design intended for widespread use in eyeglasses and contact lenses. In addition, this culture model allows RPE cells to accumulate A2E in vitro to levels, which are comparable to that present in the eye as determined by high-performance liquid chromatography quantitation.[21,36]

METHODS

Cell Culture

A human adult RPE cell line (ARPE-19; American Type Culture Collection, Manassas, VA) was grown in Dulbecco Modified Eagle Medium (Cellgro, Mediatech, Herndon, VA) with 10% heat-inactivated fetal calf serum (Invitrogen Corporation, Carlsbad, CA), 2 mM glutamine (Invitrogen), 0.1 mM minimum essential medium non-essential amino acid solution (Invitrogen), and gentamicin sulfate (10 μg/ml) in eight-well plastic chamber slides (Lab-Tek Nunc, Rochester, NY). We have shown that this human RPE cell line is devoid of endogenous A2E.[36]

A2E Synthesis and Accumulation in RPE

A2E was prepared from all-trans-retinal and ethanolamine (2:1 molar ratio) as previously described.[18] A2E is stored as a stock solution in dimethyl sulfoxide and is kept at −80°C in the dark. For uptake into confluent cultures of ARPE-19 cells, A2E was delivered in culture medium at 10 μM concentration and 300 μL volume per well. Fresh A2E was delivered twice a week and accumulation proceeded for 2 weeks. All experiments included A2E-free cells. By quantitative HPLC measurement, this protocol yielded 2130 ± 108 pmole/10^6 cells (mean ± SD, based on four measurements). The autofluorescence of cell-associated A2E was also monitored by epifluorescent illumination under a microscope (Axioplan 2 Imaging; Carl Zeiss, Thornwood, NY).

Polycarbonate Filters

Injection-molded polycarbonate lens samples were prepared from resin mixed with the dye perylene ($C_{20}H_{12}$), a polycyclic aromatic hydrocarbon (High Performance Optics). Perylene was present at concentrations of 1.0, 1.9, 3.8, 7.5, 15, 35 parts per million (ppm). Measured in cyclohexane, perylene absorbs in the range from 375 to 450 nm with absorbance maxima at 410 and 436 nm. Transmission spectra of polycarbonate containing perylene at a range of concentrations were measured with an Ocean Optics spectrometer (Ocean Optics, Dunedin FL). Baseline (background; lamp-off) measurement was obtained at the start of the experiment and the reference (lamp-on) condition was recalibrated before each sample measurement (Fig. 1).

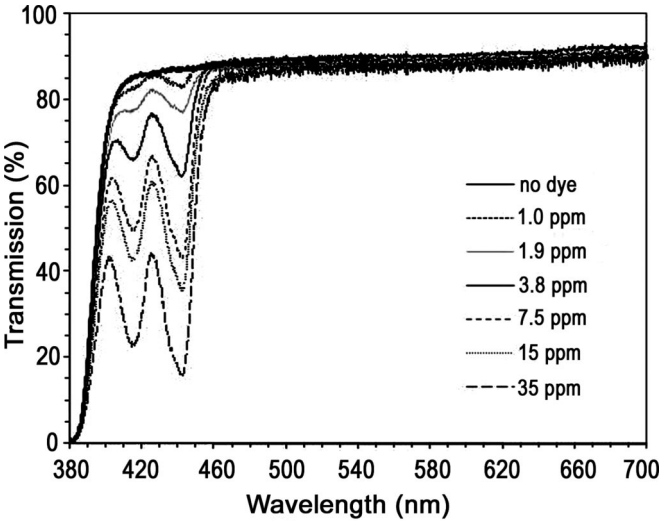

FIGURE 1.
Transmission spectra of polycarbonate filters containing various concentrations (ppm) of perylene dye.

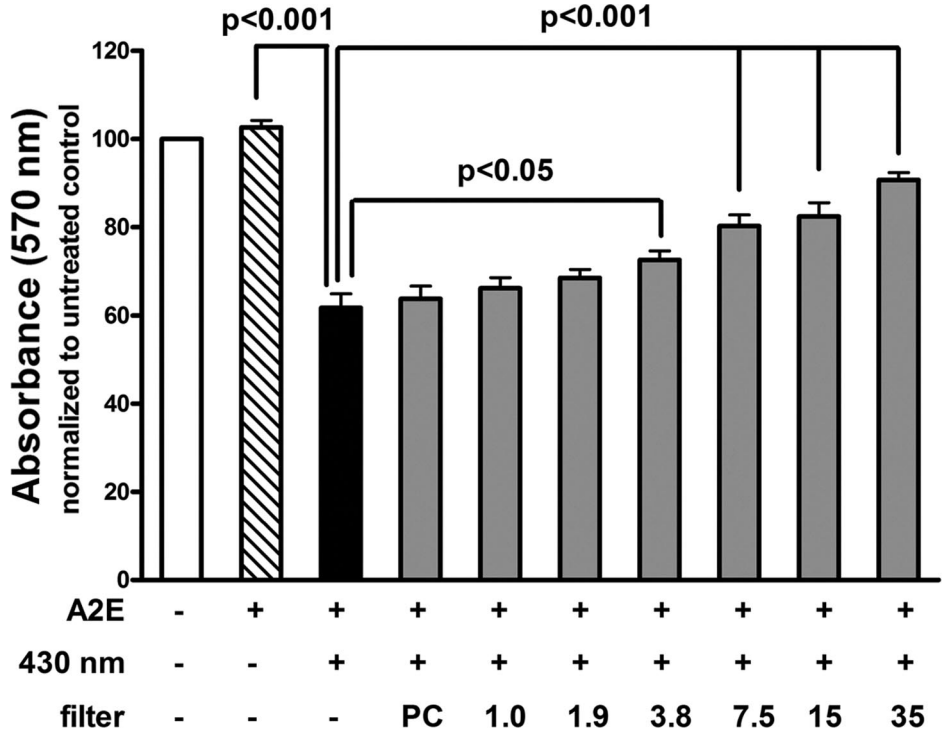

FIGURE 2.

Cell death in irradiated (430 nm) cultures of ARPE-19 cells that had accumulated A2E. Filters containing variable levels of dye (1.0, 1.9, 3.8, 7.5, 15, and 35 ppm) were placed in the light path. ± indicates presence and absence of condition. Cell death was quantified by MTT assay; absorbance values were normalized to untreated control samples. Mean ± SEM of five experiments.

Irradiation and Use of Filters

ARPE-19 cells growing in eight-well plastic chamber slides were exposed to either a light line delivered from a tungsten halogen source (430 ± 20 nm; 8 mW/cm^2; 20 min for fluorescence exclusion assay) or to the entire area of the well (1 mW/cm^2, 30 min for MTT assay). These wavelengths are consistent with the excitation spectrum of A2E[21] and light of this wavelength reaches the RPE in vivo.[37] To test for protection from light damage by the lens material, the latter was placed within the light path. The short-wavelength light-absorbing polycarbonate filters (2.5 × 4 cm) (1.0, 1.9, 3.8, 7.5, 15, and 35 ppm) described above and a polycarbonate filter containing no dye (PC) were placed in the light path.

Cell Viability Assays

Cytotoxicity after 24 h was measured by a well known metabolic (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) colorimetric microtiter assay (Roche Diagnostics Corporation, Indianapolis, IN).[38] The assay is based on the ability of healthy cells to cleave the yellow tetrazolium salt MTT to purple formazan crystals. The assay is based on the ability of healthy cells to produce reducing equivalents (NADH or NADPH) that via mitochondrial reductase enzymes, cleave the tetrazolium salt MTT (yellow) so as to form purple formazan (purple) crystals; production of the latter is proportional to the number of viable cells in the culture. To perform the MTT assay, 20 μl of MTT labeling reagent (Roche Diagnostics Corporation, Indianapolis, IN) is added to 0.2 ml of culture medium in each well and after a 4 h incubation, another 200 μl of solubilization solution was added to each well for an overnight incubation. After centrifugation at 13,000 rpm for 2 min, supernatants were measured spectrophotometrically at 570 nm absorbance. A decrease in the absorbance (570 nm) of reduced MTT is indicative of diminished cellular viability. Values are presented as corrected absorbance calculated as absorbance of treated samples/absorbance of untreated control × 100. In each experiment, one well was assayed per experimental condition. Data were based on five independent experiments.

We also counted non-viable cells after labeling by a fluorescence exclusion assay that allowed for the labeling of apoptotic nuclei because of a loss of plasma membrane integrity during the latter stages of cell death. Accordingly, 8 h after blue light exposure, the nuclei of dead cells were stained with the membrane impermeable dye Dead Red (Molecular Probes; 1/500 dilution, 15 min incubation) and all nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI). In all experiments, replicates were assayed by counting DAPI-stained and Dead-Red stained nuclei in at least five microscopic fields within the area of illumination in each well; three wells were assayed per experiment. Values are presented as Dead-Red-stained nuclei/DAPI stained nuclei × 100. Data were based on three experiments.

Statistical Analysis

Data were expressed as mean ± SEM. To test for differences among all the groups (A2E + blue light; A2E + blue light + lens; A2E only; A2E free), a one-way analysis of variance was applied

using statistical software (Prism, GraphPad Software); $p < 0.05$ was considered statistically significant.

RESULTS

As was reported earlier,[21,22,24,38] irradiation of ARPE-19 cells that had previously accumulated A2E lead to a loss of cell viability. Measured by MTT assay and presented as percent cell viability normalized to the untreated control, the reduction in viability in the present experiments was 40% (Fig. 2, black bar). However, illuminated RPE cells that did not contain A2E remained viable.

When a series of filters representing a gradient of blue light-absorbing dye were placed in the light path, protection of 430 nm irradiated A2E-laden RPE was observed. Moreover, the extent of protection was dependent on the concentration of the dye. By MTT assay, a statistically significant difference ($p < 0.05$) between irradiation in the absence of a filter and irradiation in the presence of a filter was reached at a concentration of 3.8 ppm. Protection, measured as an increase in cell viability, was 18% with the 3.8 ppm filter, increased to 30% with the 7.5 ppm filter, and reached 47% with the 35 ppm filter. The modest decrease in cell death (3%) observed with the dye less filter (Fig. 2, PC) was due to reduction in light transmission (~15%) associated with polycarbonate alone.

Similar findings were observed when cell death was quantified by fluorescence staining of the nuclei of membrane compromised cells although the gradient of change was more pronounced (Fig. 3). Here, the 1.9 and 3.8 ppm filters provided 23% and 28% increases in percent viable cells, respectively, whereas protection reached 50% with the 35 ppm lens.

DISCUSSION

The notion that light may contribute to the retinal cell decline leading to age-related macular degeneration (AMD) originated several years ago.[39,40] Indeed, some epidemiological studies investigating a possible relationship between light exposure and AMD have demonstrated a relationship. The Chesapeake Bay Waterman Study found that a small number of subjects with advanced AMD, including geographic atrophy, had the highest estimates of blue light exposure during the 20-year period leading up to the study.[41,42] Another population-based cohort study, the Beaver Dam Eye Study, reported at baseline, and at 5- and 10-year follow-up that levels of sun exposure from the second decade of life forward was related to incidence of early AMD.[43–45] The Age-Related Eye Disease Study acknowledged indications that sunlight exposure was a risk factor although the association did not reach significance (report 3).[46] One problem in conducting studies such as these is the difficulty inherent in estimating sunlight exposure. Indeed two small studies, an Australian case-control study and the Pathologies Oculaires Liées à l'Age (POLA) study did not find evidence of a relationship.[47,48] However, recently, the European Eye Study reported a significant association between lifetime blue

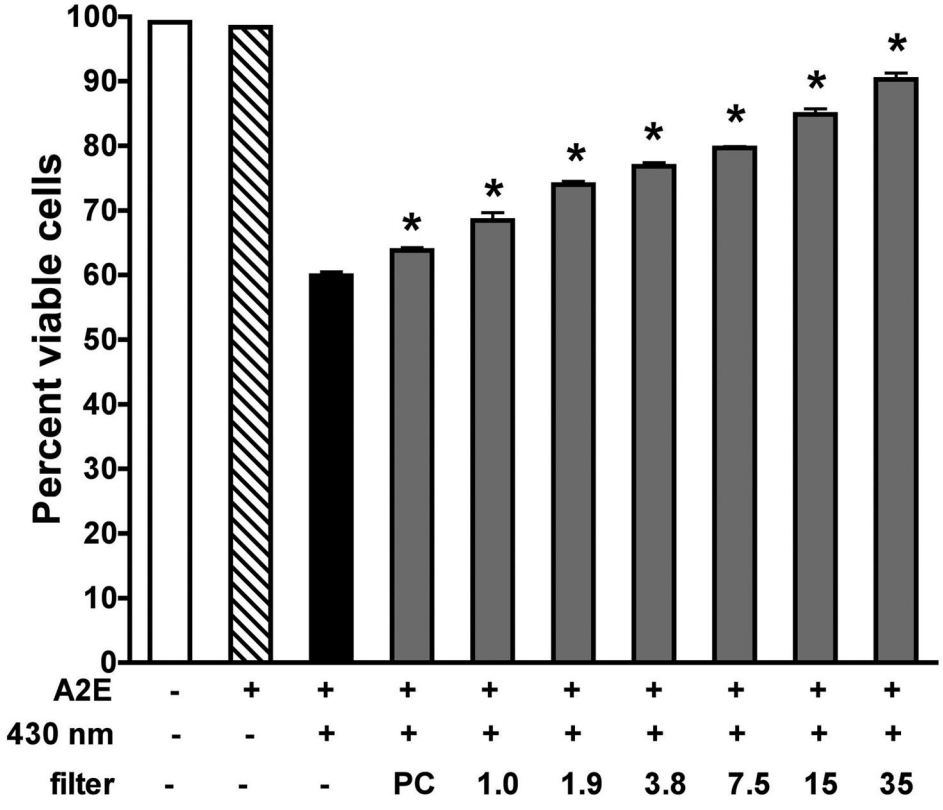

FIGURE 3.

Quantitation of viable RPE cells after A2E accumulation and blue light illumination (430 nm), with and without a blue-light absorbing filter placed in the light path. Filters contained variable levels of dye (1.0, 1.9, 3.8, 7.5, 15, and 35 ppm) or no dye (PC). The percent of viable cells was determined by labeling all nuclei with DAPI and the nuclei of non-viable cells with a membrane impermeable dye. The data were normalized to values determined in the absence of a filter and are presented as percent viable cells. *$p < 0.001$ when compared with A2E, 430 nm. Mean ± SEM of three experiment with triplicate wells in each experiment.

light exposure and neovascular and early AMD in individuals exhibiting low levels of vitamin C, zeaxanthin, vitamin E and zinc; the number of a patients with geographic atrophy was not sufficient for analysis.[49]

The potential significance of light to retinal pathology is not necessarily limited to AMD. Autosomal recessive Stargardt macular degeneration, a disorder of juvenile onset caused by *ABCA4/ABCR* gene mutations, is characterized considerably by accelerated RPE lipofuscin formation.[50,51] Indeed, RPE lipofuscin is considered the cause of RPE atrophy in Stargardt disease and the wearing of yellow-tinted sunglasses is advocated as a prophylactic approach to this form of vision loss. The progression of retinal degeneration in some forms of retinitis pigmentosa may also be aggravated by light exposure. Other diseases to be considered in this regard include dominant Stargardt-like macular degeneration as a result of mutations in ELOVL4 and the vitelliform macular dystrophies caused by mutations in VMD2, the gene encoding bestrophin-1. All these disorders are associated with abnormalities in RPE lipofuscin.

The damaging effects of A2E on the RPE cell are likely attributable, in large part, to two properties of the compound: an amphiphilic structure that confers detergent-like activity and photooxidative processes. The relative importance of these two properties is not known at this time. It has been shown that A2E does not have direct effects on lysosomal enzyme activity.[52] The proposal that A2E impacts on lysosomal degradative functions arose from the assumption that A2E enters lysosomes in a deprotonated state and within the lysosomal interior becomes protonated thereby decreasing lysosomal pH.[53] However, A2E is a quartenary pyridinium salt that cannot be deprotonated or reprotonated.[17,36] However, some effects of A2E on cells[54] could be attributable to the fact that A2E is an amphiphilic molecule that exhibits detergent-like activity that could be exerted at intracellular membranes.[36,53,55,56] Interestingly, another bisretinoid lipofuscin pigment, all-trans-retinal dimer-PE, is a Schiff base with an imine nitrogen that is protonated. This constituent of RPE lipofuscin has the potential to undergo pH-dependent deprotonation within lysosomes.[26]

The natural crystalline lens in the human eye yellows with age because of protein glycosylation and to the deposition of oxidation products of tryptophan.[57] Attention has been given to the possibility that the attenuation of short wavelength blue light that accompanies the color change in the lens, protects RPE cells from lipofucin-associated photochemical damage.[58–61] Several areas of investigation implicate RPE lipofuscin in the pathogenesis of AMD. For instance, photooxidation products of A2E and all-trans-retinal dimer have been shown to activate complement.[62,63] This finding is significant because dysregulation of complement activation is considered to underlie the observed associations between genetic variants in complement factors and susceptibility to AMD.[64–67] AMD has onset in the elder years, but it develops progressively over considerable time before diagnosis. Complement activation triggered by photooxidation products of RPE bisretinoid lipofuscin could begin early in life and generate low-grade inflammatory processes that gradually predispose the macula to disease. Were this is the case; consideration could be given to the use of light-filtering lenses by susceptible individuals at an early age. The benefits of an intervention such as this would need to be tested in clinical trials.

The series of filters in this work provided dye dependent protection against blue light damage in a culture model. In this way, mostly colorless filters selective filtering method to protect the human eye from not only UV light but also high energy visible light damage. The clinical benefits of this filtering would have to be determined by appropriately designed studies.

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Janet R. Sparrow
Columbia University
Department of Ophthalmology
630 West 168th Street
New York, New York 10032
e-mail: jrs88@columbia.edu